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THE INFLUENCE OF HIGH FAT DIET ON HYPOTHALAMIC NEURAL ACTIVITY: AN EXAMINATION OF C-FOS, α-MSH and NPY IMMUNOREACTIVITY AND LEPTIN RECEPTOR m-RNA STATEMENT IN HYPOTHALAMUS

by

YongLin Zhang

A thesis submitted in part fulfilment of the requirements for the award of the degree

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2000

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ABSTRACT

This thesis examines the influence of a high-fat diet on hypothalamic neural activity. The hypothalamus plays an essential role in regulating body weight. Peripheral tissues, particularly fatty tissue, send signals such as leptin to the hypothalamus to activate a neural network in the brain that regulates food intake and energy balance. This neural network is coded with specific neurotransmitters and receptors. Hypothalamic Neuropeptide-Y and alpha-MSH (alpha-Melancortin stimulate hormone) systems are critically involved in this regulation of body weight.

Twelve C57BL/6J(+/+) male mice were fed with two different diets separately: a high-fat diet or a low-fat diet. Each of these two groups of mice were sacrificed at different time: after one or eight weeks feeding. The aim of this study is to search for the altered hypothalamic neuronal activity in these mice.

The c-Fos immunoreactivity significantly increased in the lateral hypothalamus (LH) (+61%), dorsomedial hypothalamic nucleus (DMH) (+40%) and arcuate nucleus (Arc) (+57%) in high-fat diet mice after eight weeks feeding. However, no significant changes in c-Fos immunoreactivity were found in the mice fed with a low-fat diet. The NPY immunoreactivity was significantly increased in the ventromedial hypothalamic nucleus (VMH) (+98%), LH (+39%), DMH (+27%), paraventricular nucleus (PVN) (+20%) and perifornical nucleus (PeF) (+39%) in the mice fed a high fat diet for eight weeks, although no significant alteration in NPY immunoreactivity was detected after one week. The influence of the high fat diet on alpha-MSH immunoreactivity was specific to the DMH (+37%) where it was significantly increased after eight weeks. In addition, a moderate increase in alpha-MSH immunoreactivity was found in the LH

(+38%) after eight weeks in high-fat dietary mice. There was no detectable change in the level of leptin receptor mRNA expression in all four groups of mice.

The results of this study indicate that a high fat diet alters the level of hypothalamic NPY and alpha-MSH immunoreactivity in specific sites that may contribute to the development of obesity or excess-fat storage. Understanding the central mechanisms of body weight control is only at its early stages. Further study, not only in animals but also in humans, on the specific receptors of NPY and alpha-MSH needs to be pursued.

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ABBREVIATIONS

3V	third ventricle
ACTH	adrenocorticotropic hormone
α-MSH	alpha-melanocyte stimulating hormone
Arc	arcuate nucleus
CA1-3	fields Cal1-3 of ammons horn
Cg	cingulate gyrus
CNS	central nervous system
CORT	corticosterone
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DG	dentate gyrus
DIG	dentate gyrus
DMH	dorsomedial hypothalamic nucleus
DNA	deoxyribonucleic acid
HF	high-fat
Ig	immunoglobulin
LF	low-fat
LH	lateral hypothalamus
LHA	lateral hypothalamic area
LR	leptin receptor
MSH	melancortin stimulate hormone
MC1-R	melanocortin-1 receptor
MC2-R	melanocortin-2 receptor
MC3-R	melanocortin-3 receptor
MC4-R	melanocortin-4 receptor
mRNA	messenger Ribonucleic acid
NAS	nickel ammonium sulphate
NBT	4-nitro blue tetrazolium chloride
NPY	neuropeptide Y

PBS	phosphate buffer solution
PeF	perifornical nucleus
Pir	pirifornical cortex
POMC	proopiomelanocortin
PVN	paraventricular hypothalamic nucleus
VMH	ventromedial hypothalamic nucleus

Chapter 1: Introduction and aims

1.1 Overview of obesity

Obesity can be seen in all human societies and is wide spread in the animal kingdom. In a variety of animal and human studies, it has been shown that obese individuals have a greater risk for cardiovascular disease and type II diabetes mellitus (Larsson B et al., 1984). The animal model for obesity can be induced by high fat diet (Schemmel R et al., 1970).

1.2 Diet and obesity

The amount of food eaten is an essential component related to body weight gain. Food provides the energy that is required for metabolism and for the storage of nutrients for later use. Thus, the proper control of food intake is essential for maintaining a healthy body composition and avoiding obesity. In energetic terms, excess fat deposition must result from an imbalance between energy intake and out-put. Thus, obesity might be considered as a very simple problem of defective regulation of energy balance: energy intake exceeds energy expenditure (Bradford B.Lowell, 2000).

Energy balance is determined by macronutrient intake, energy expenditure, and partitioning in nutrient storage. Positive energy balance over weeks and months will result in weight gain whereas negative energy balance will have the opposite effect (George Bray, 1997). It is useful to analyses energy expenditure from a thermodynamic perspective. Obesity is the result of energy imbalance over time and, owing to its cumulative nature, it can develop when energy intake exceeds energy expenditure by

only a small margin over a long term. Total body energy expenditure represents the conversion of oxygen and food (or stored forms of energy such as fat, glycogen and protein) to carbon dioxide, water, heat, and work on the environment (Bradford B. Lowell, 2000).

Increasing the fat content of the diet presented to laboratory rodents usually results in excessive weight gains. Previous research in Surwit's laboratory has demonstrated that the C57BL/6J(B/6J) mouse has a predisposition to develop severe obesity if placed on a high-fat diet(Surwit, 1988). In another experiment in his lab the obesity-prone B/6J mice were placed on one of four diets which contained either high-fat or low-fat. After 4 months, all of the animals on the high-fat diets had gained more weight than animals on the low-fat diets(Brownlow, 1996).

Vice versa. In Surwit's research group, the other interesting experiment was to determine if obesity and diabetes in the C57Bl/6J mouse could be completely reversed by reducing its' dietary fat content. After 4 months, C57Bl/6J mice consumed more calories on a high-fat diet than on a low-fat diet, and they showed a higher feed efficiency (FE= weight gained/calories consumed) on the high/fat diet versus the low-fat diet. Hyperglycemia, hyperinsulinemia, and increased adiposity were apparent in B6 mice after 4 months on the high-fat diet regardless of whether the diet begun at weaning or 4 months later. Corelational analyses showed that adiposity was strongly related to both insulin and glucose levels in B6 mice, but only moderately reversed. Adiposity, fasting glucose, and fasting insulin values in these mice were equivalent to those in B6 mice of the same age that had spent 8 months on the low-fat diet. These studies have demonstrated that

dietary factor plays an important role in the development of diet induced obesity. Further, developed diet-induced obesity can be reversed in C57Bl/6J mice (Priti, 1998).

The magnitude of this effect (that a high-fat diet induces obesity or a low-fat diet reverses obesity) is dependent on other factors, such as the duration of the experiment and the genetic background of the animals, with some strains being virtually resistant to obesity (Schemmel and Mickelsen, 1973; Miller, 1979). High-fat diets may stimulate energy intake because of their greater palatability compared with standard pelleted diets and/or because of the increased energy density. However, several groups have reported greater body fat gains in rats and mice without any increase in energy intake (Lemonnier, 1972; Herberg et al, 1974), indicating that energetic efficiency is increased. One important result of a high-fat intake is that most of the fat deposited in the body can be derived directly lipid with a much lower energy cost (6kJ/g deposited) than de novo lipogenesis from carbohydrate sources (14kJ/g), thereby increasing the efficiency of the gain. In general, the metabolic effects of high-fat diets are extremely complex.

Some humans appear to resist fat gain with overeating, whereas others readily store excess fat. These subjective observations have been confirmed by a small number of clinical studies that document a several fold interindividual variation in fat accumulation with overfeeding (Sims E.A. et al, 1973; Bouchard C. et al, 1990; Diaz E.O. et al, 1992). Further, higher thermogenesis that allows some individuals to resist weight gain, despite over-eating has not been identified.

1.3 Hypothalamus and Obesity

Hypothalamus plays an important role in the regulation of food intake and energy expenditure as well as temperature, heart rate, blood pressure, and blood osmolarity and food and water intake. It also participates in the regulation of homeostasis by receiving information directly from the internal environment, and by operating directly on the internal environment.

The hypothalamus exerts its influence on both the internal and external environments through three major systems: the endocrine system; the autonomic nervous system; and motivation.

Since food intake activity is a behavior, it must be mediated by the brain. The amount of food intake is controlled by internal signals. For example, if the rats are force-fed for a period of time until they become obese, the rats will reduce their food intake, when allowed to do so, and will bring their body weight back down to normal levels (Cohn C et al., 1962). Conversely, when the rat's food intake is restricted for a period of time so that they lose weight and become thin, they will increase their food intake, when they are allowed to do so, and will again bring their body weight up to normal levels. Although the study clearly shows that there are internal mechanisms that adjust daily food intake following changes in body weight, it does not show how the task is accomplished. During force feeding and starvation there are changes in the degree of stimulation of the gastrointestinal tract, in the internal metabolic pathways of the liver and other organs, and in the storage of nutrients in muscle and fat. When force feeding or starvation stops, any or all of these changes could be responsible for generating an

internal signal that brings cumulative food intake and body weight back to normal levels.

Compare to 10 years ago, , there has been a large progress in understanding the neural pathways involved in the monitoring of carbohydrate and fat metabolism. The recent work by Ritter's group has provided important new evidence of the connections between peripheral physiology and regions of the brain concerned with feeding. Their work suggests that both fat and carbohydrate oxidation are separately monitored by the CNS, and that these signals are interfaced within the brain to monitor overall fuel status (Ritter S et al., 1994).

There is now an extensive and growing understanding of both central and peripheral hormones, peptides, and others (Leibowitz S.F., 1995).

In studying the brain, it is essential to identify the precise areas and cell groups of the brain that are critically involved in body weight regulation. There are several specific nuclei that play an important role in maintaining energy balance.

The lateral hypothalamus (LH) is like a city train station with many nerve fibers passing through local circuit and sensory cell groups . The complexity of the LH is reflected in the scientific history of this region, which Stellar characterized as a "feeding center" (Teitelbaum P. and Stricker E.M., 1994). The LH contains glucose-sensitive cells, feeding related neurons with monoamine and peptide receptors, nerve fibers involved in feeding reward, and fibers of passage subserving motive functions. Some LH fibers support stimulation-induced eating and reinforce self-stimulation.

Many electrophysiological studies have reported that the VMH and the LH are reciprocally linked and inhibitory to each other (Roesch T. et al., 1984). It has also been shown that one third of VMH neurons increase their activity when glucose is applied, while neurons in the LH are specifically inhibited (Oomura Y. et al., 1973).

In the last two decades, exciting developments have provided extensive new information, extending this framework into a neuronal network including the dorsomedial hypothalamic nucleus (DMH), paraventricular hypothalamic nucleus (PVN), arcuate hypothalamic nucleus (Arc), and other nuclei.

The direct VMH-LH connections have been reported as (Guillery R.W., 1957; Millhouse O.E., 1973; Saper C.B. et al., 1979) indirect connections between VMH and LH, which have been shown to occur via the DMH (Luiten P.G.M. et al., 1987; Luiten P.G.M. et al., 1980; Ter Horst G.J. et al., 1986). Thus, the main stream of connections within the hypothalamus have been proposed to run from the LHA and VMH to the PVN via the DMH (Luiten P.G.M. et al., 1987). This brings the DMH in a position to play an integrative role in overall homeostasis by functioning both as a regulator of the VMH-LH interaction, and as a modulator of the neuroendocrine output of the PVH (Bernardis, L.L., 1998). The perifornical region (PeF) within the LH may be specialized for potentiating feeding reflexes, and reinforcing voluntary motor functions of ingestive behavior.

Using electron microscopic techniques, Zaborsky et al found that, following transaction at the level of the posterior hypothalamus-mamillary body in the rat, direct pathways could be traced from the site of transaction to the ARC, VMH,

and DMH, and even as far rostral as the PVN. Recent evidence suggests that DMN, together with the VMN, and the arcuate nucleus (ARC) of the hypothalamus, may be part of the circuitry that is responsive to the feedback signal from adipose tissue by the hormone leptin (Bernardis L. L., 1998).

1.4 Letpin and Leptin Receptor

1.4.1 Leptin

It has long been assumed that food intake was regulated by the adipose tissue mass through a feed-back mechanism which inhibits or stimulates hypothalamic nuclei (Friedman J.M. et al., 1992). One signal protein is now known as leptin produced by the gene named as obese (ob) gene (Zhang Y. et al., 1994).

The obese gene is associated with an obese phenotype (Friedman J.M. et al., 1992), which manifests itself as enhanced lipid deposition in adipose tissue, excessive feeding, attenuated energy metabolism, and type II diabetes.

The theory that body fat mass is involved in the regulation of body weight through a feedback loop was proposed more than 40 years ago. Parabiosis experiments provided evidence for a hormonal factor that acts as a satiety signal (Hervey G.R. et al., 1959). It was shown that ob/ob mice are deficient in producing this signal (Hausberger F.X., 1958), whereas db/db are insensitive (Coleman D.L. et al., 1969).

In rodent models of obesity, leptin plays a key regulatory role in energy balance via the hypothalamus with its actions mediated through specific leptin receptors. Intraperitoneal injection of leptin in such animals results in a marked

reduction in food intake, increased energy expenditure and substantial weight loss (Campfield et al., 1995). Injections of leptin into mutant ob/ob mice normalises most aspects of the obesity/diabetes syndrome (Campfield et al., 1995). Furthermore, injections of leptin into the brain are efficient, indicating that the major site of action of leptin is within the central nervous system.

In addition to its metabolic effects, leptin has a strong influence on a number of endocrine systems. In male mice, it blunts the starvation-induced marked decline of luteinizing hormone, testosterone, thyroxine, and the increase in adrenocorticotrophic hormone (ACTH) and corticosterone. In female mice, leptin prevented the starvation-induced delay in ovulation (Ahima R.S. et al., 1996). Ob/ob female mice are sterile. This defect can be corrected by leptin administration (Chehab F.F. et al., 1996).

Leptin is exclusively expressed and secreted by differentiated adipocytes (Rentsch J. et al., 1996). The major determinant of circulating leptin concentration in both rodents and humans is fat mass (Frederich R.C. et al., 1995; Maffei M. et al., 1995; Considine R.V. et al., 1996). Leptin levels have been measured in various obesity models. The circulating plasma levels of leptin correlates with the measurements of fat mass. This suggests that the leptin production is proportional to the fat mass. Furthermore, food deprivation and weight reduction are known to reduce leptin levels, suggesting that there are controllers for the leptin production except fat mass (Frederich et al., 1995). For example, the levels of leptin of normal adults (n=10) decreased to about 50% after 24 hours and further declined to about 30% after 33 hours. Thereafter leptin levels remained stable upon continuous

fasting for 3 days (Blum et al., 1997). Based on these observations, it is suggested that leptin's normal physiological role might be to serve as an emergency signal for a critical level of fat store depletion (Spiegelman and Flier, 1996).

Leptin levels increase exponentially with body mass index (BMI) or the percentage of body fat (Frederich R.C. et al., 1995; Maffei M. 1995; Considine R.V. et al., 1996). It has been reported that the correlation of leptin levels with BMI in humans shows an almost identical relationship in boys and girls up to teenage stage (Blum W.F. et al., 1997).

A strong stimulating effect of insulin on leptin statement, and on leptin levels was shown in vitro (Saladin R. et al., 1995; Slieker L.J. et al., 1996; Leroy P. et al., 1996), and in vivo in both rodents and humans (Saladin R. et al., 1995). Glucocorticoids increase leptin levels in vivo and have a direct stimulatory effect on leptin statement in vitro (Slieker L.J. et al., 1996).

The various effects of leptin appear to be mediated through its action on the hypothalamus. In particular, modulation of neuropeptide Y(NPY) is pivotal.(Campfield L.A. et al., 1996; Rohner-Jeanrenaud F. et al., 1996) Leptin suppresses NPY statement and secretion in the arcuate nucleus (Schwartz M.W. et al., 1996). Leptin that inhibits food intake and energy expenditure was thought to function through inhibition of the hypothalamic neuropeptide Y (NPY), a stimulator of food intake.

The picture of the sequence adipocyte $\Rightarrow$ leptin secretion $\Rightarrow$ leptin action on the hypothalamic receptor $\Rightarrow$ decreased NPY synthesis $\Rightarrow$ low NPY secretion $\Rightarrow$ lowers food intake.

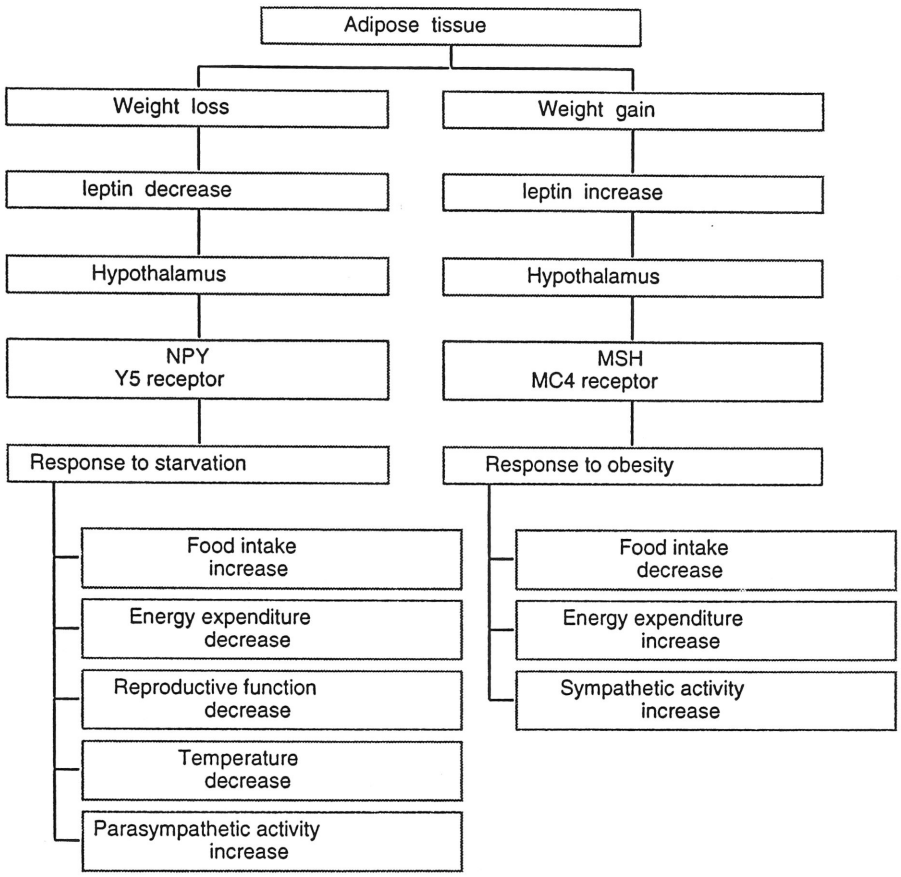

Figure 1.1: Pathway that leptin activates hypothalamic neural activity regulating body weight.

1.4.2 Leptin receptor

The leptin receptor (Ob-R) is a single membrane-spanning protein. It exhibits considerable homology with the glucoprotein 130 subunit of the interleukin 6 receptor, and belongs to the cytokine class I receptor family(Campfield et al., 1995). Ob-R statements have been demonstrated in rodents in various areas of the central nervous system, particularly in the hypothalamus (Tartaglia L.A. et al., 1995; Chen H. et al., 1996; Lynn R.B. et al., 1996; Considine R.V. et al., 1996), and also in multiple

peripheral tissues such as lung, kidney, liver, skeletal muscle, heart, testis and adipose tissue. (Tartaglia L.A. et al., 1995; Lee G.H. et al., 1996).

Leptin receptors have five isoforms: Ob-Ra,b,c,d,e. All isoforms share the same extracellular domain. The letpin receptor (b) is defective in db mice, while all other isoforms are unaffected. Disruption of the letpin receptor is sufficient to produce the entire spectrum of deficits seen in mutants in which all forms of the receptor are disrupted.

Leptin receptor (a) can be found in all peripheral tissues and is capable of leptin-mediated signaling, but much less effective than the full length receptor (Murakami et al., 1997). Leptin receptor (a) is also found in the choroids plexus and is reported to be involved in transporting leptin from blood stream to brain (Lee et al., 1996). Leptin receptor (e) is found in blood stream and is thought to be as a carrier for leptin (Sinha et al., 1996).

1.4.3 High-fat diet and leptin resistance

It is reported by Van Heek's research (Margaret Van Heek, 1997), that the obese mice were induced by exposure to a 45% fat diet, whereas the mice responded to peripherally administered letpin, leptin resistance developed after 16 days of high fat diet. After 56 days the mice became resistant to peripherally administered leptin, but responsed to the central administration of leptin. This experiment demonstrates that, in a diet-induced obesity model, mice exhibit resistance to peripherally administered leptin, while retaining sensitivity to centrally administered leptin.

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Similar results were reported by our group (Lin et al., 2000). After 1 week of feeding, the mice fed a high-fat diet were sensitive to intraperitoneal injection of leptin. After 8 weeks of feeding, the same mice fed a high-fat diet were insensitive to intraperitoneal injection of leptin, but sensitive to intracerebroventricular injection. of leptin. Furthermore, after 19 weeks of feeding, the mice were insensitive to i.c.v. leptin, but were sensitive to a high dose of i.c.v. leptin. Therefore, it is suggested that leptin resistance can be divided into three stages. In the early stage mice were sensitive to exogenous leptin, in the middle stage mice had an increase in leptin production and still retained central leptin sensitivity, in the late stage mice had a reduction of central leptin sensitivity.

Leptin resistance is defined as an increase in plasma leptin levels accompanied by an increase in body weight gain and food intake, but the precise mechanism inducing leptin "resistance" is currently not clear.

1.5 Alpha Melanocyte stimulating hormone (α-MSH)

The melanocortin peptide is another neuropeptide that is derived from the gene called pro-opiomelanocortin. The POMC gene expresses the proteins of adrencorticotrophin (ACTH) and melanocyte-stimulating hormones (MSH).

The POMC gene is expressed in the hypothalamic arcuate nucleus, solitary nucleus, and anterior lobe of the pituitary gland (Cheung C.C. et al, 1997). The POMC gene was cloned in the early 1980's (Drouin J., 1980; Nakanishi S., 1981). Besides in the brain, POMC statement has also been detected in several peripheral

tissues including the skin, pancreas and testis (Kathleen G. Mountjoy and Jencia Wong, 1997).

POMC neurons of the arcuate nucleus project to a number of brain areas including several hypothalamic nuclei, including the median eminence, the septum, the periventricular region of the thalamus, and the amygdala (Eskay et al., 1979). Also, POMC-producing neurons of the nucleus solitarius innervate the medulla and the spinal cord (Joseph S. A. and Michael G. J. et al., 1988).

The POMC precursor is processed to form the melanocortins α-MSH and α-MSH, which stimulate pigmentation via their activities on skin melanocytes. α-MSH is a 13-amino-acid pro-opiomelanocortin (Lipton J. M., 1988).

The alpha-Melanocortin stimulating hormone (α-MSH) appears to be important in the regulation of energy balance and body weight, influencing both food intake and sympathetically mediated thermogenesis.

An intracerebroventricular injection of α-MSH produces a decrease in spontaneous feeding in adult rats (Poggioli R. and Vergoni A. V. et al., 1986; Vergoni A. V. and Poggioli R. et al., 1990).

It has been previously proposed that a defect in α-MSH acetylation could contribute to obesity and diabetic phenotypes, based on the characterization of a variant 'yellow' mouse. The agouti (a) locus in mice makes a protein that switches the melanocytes from black melanin synthesis to yellow, and is recognized most widely for its role in the regulation of coat pigmentation. A loss of this agouti gene protein product results in black pigment only, and an overproduction in yellow (A^{vy}/a). 'Yellow' mice with mutations at this locus are associated with an

all-yellow coat color, and effects of obesity and diabetes (Kathleen G. Mountjoy and Jencia Wong., 1997).

1.6 Neuropeptide Y in body weight control

Neuropeptide Y(NPY) research began with the discovery of the first pancreatic polypeptide (PP) isolated from the avian pancreas (aPP) (Larsson et al., 1975). After several years, Tatemoto and co-workers isolated two PP-like peptides, which they named Neuropeptide Y (NPY) and peptide YY (PYY), respectively from porcine brain and gut extracts (Tatemoto 1982a, 1982b; Tatemoto et al., 1982).

Neuropeptide Y is one of the most abundant peptides found in the CNS in all mammalian species studied (Stanley B.G. et al, 1985). NPY-like immunoreactivity is found in large quantities in various nuclei of the hypothalamus, throughout most cortical areas, in the septum, hippocampus, olfactory bulb and striatum, as well as, in certain mesencephalic and mesencephalic nuclei of the rat and human brain (Adrian et al., 1983). In situ hybridization revealed that the localization of NPY mRNA correlated well with the distribution of NPY-like immunoreactivity, which provides strong evidence that NPY-like material is synthesized, processed and stored in these neurons of Arc (Chan-Palay et al., 1988; Terenghi et al., 1987).

The highest amounts of NPY receptor binding sites in the brain are located in the hippocampus and the amygdaloid-hippocampal area of all mammalian species studied so far (Busch-Sorensen et al., 1989; Giardino et al., 1989). Neuropeptide Y-like perikarya and fibers are present in high densities in several hypothalamic nuclei; often being co-localized with noradrenaline (Chronwall et al.,

1985). Neuropeptide Y receptor sites are also found in the hypothalamus, although in lesser concentrations than in the cortical and the hippocampal areas (Martel et al., 1986). The hypothalamus is known to receive a rich innervation of NPY-immunoreactive nerve endings, and the hypothalamic paraventricular nucleus (PVN) is particularly dense with NPY terminals (Mutt, V. et al., 1989). These fibers originate either from catecholamine-containing neurons in the medulla and dorsal pons, or from noncatecholaminergic neurons in the arcuate nucleus (Arc) (Martel J.C. et al., 1986).

Neuropeptide Y is a powerful stimulant of food intake. When injected into the central nervous system of rats, neuropeptide Y results in an obesity syndrome closely resembling the phenotype of either leptin deficient ob/ob mice or leptin resistant db/db mice (Zarjeveski N. et al., 1993; Chua S.C. et al., 1991). However, being deficient of NPY, it was also reported that mice have normal food intake and body weight (Jay C. Erickson et al., 1996).

1. The effects of NPY on endocrine systems

Neuropeptide Y has strong effects on the release of certain hormones, including corticosterone(CORT), aldosterone (ALDO), insulin (INS) and vasopressin (AVP), which control energy metabolism, as well as food ingestion. The PVN, which contains both the neurons that synthesize corticotropin-releasing factor (CRF) and AVP, and the steroid receptors that bind CORT and ALDO, is believed to have a primary function in regulating the secretion of these hormones (Swanson L. W. et al., 1983). When injected directly into this nucleus, NPY has a rapid stimulatory effect on the release of both CORT and AVP (Leibowitz S. F. et al.,

1988). A similar effect on the release of INS and ALDO has also been observed after NPY administration into the PVN or cerebroventricles (Abe M. et al., 1989; Fuxe K. et al., 1989).

The effects of NPY on the secretion of each of these hormones, therefore, links this peptide, to a variety of physiological systems involved in energy homeostasis.

2. Impact of NPY on metabolic processes

In addition to its endocrine effects, NPY has been shown to influence energy metabolism through effects on substrate utilization. Acute injection of NPY to the PVN preferentially stimulates carbohydrate ingestion, increases fat deposition and body weight gain while potentiating both fat and carbohydrate consumption (Menendez, J. A. et al., 1990). These findings agree with recent evidence showing that ventricular NPY administration can promote white fat lipid storage while decreasing brown fat thermogenesis by reducing sympathetic activity of interscapular brown adipose tissue (Egawa M. et al., 1991).

3. Effects of NPY on eating behavior

Studies of NPY's impact on eating behavior have revealed a potent stimulatory effect after administration into the ventricles (Clark J. T. et al., 1984; Levine A. S. et al., 1984) and after direct injection into the PVN (Stanley B.G. et al., 1985). The PVN has been closely associated with the control of feeding behavior, both in lesion studies (Leibowitz S. F., 1981; Shor-Posner G. et al., 1985) and in investigations of catecholamine neurotransmitters (Leibowitz S. F., 1988). In this nucleus, NPY and its related substance, peptide YY (0.02-1.0 nmoles), dose-

dependently increase feeding and cause rats to eat, in just 1 hour, approximately 50% of their normal daily intake (Stanley B. G. et al., 1985). The eating response induced by NPY is associated with an increase in both the rate and the duration of eating (Clark J. T. et al., 1984; Stanley B. G. et al., 1985). The response elicited by NPY in the PVN is relatively long(10 minutes), and NPY's duration of action has lasted up to 24 hours after a single injection. This characteristic allows NPY to have a particularly potent and long-term stimulatory effect on daily food intake, body weight gain and fat deposition after chronic administration (Stanley B. G. et al., 1985; Stanley B.G. et al., 1986).

In rats with macronutrient diets available, acute administration of NPY into the PVN selectively stimulates the ingestion of carbohydrate, having little or no effect on protein or fat consumption (Stanley B. G. et al., 1986). However, after chronic injections of NPY, fat, as well as carbohydrate intake, are both potent, which results in a profound increase in fat deposition and body weight gain (Stanley B. G. et al., 1989; Stanley B. G. et al., 1986).

4. Changes in endogenous NPY in response to food deprivation

Several studies have demonstrated that food deprivation causes an increase in hypothalamic NPY content (Sahu A. et al., 1988). It occurs especially in the PVN and ARC, precisely where NPY levels are found to rise just prior to the onset of the natural feeding cycle, and then sharply to decline following a brief episode of food digestion (Shor-Prosner G. et al., 1985; Tempel D. L. et al., 1989).

The major hypothalamic NPY pathway arises from the neuronal pathway located in the arcuate nucleus, which send afferent fibers to the anterior

hypothalamus, the preoptic area, the PVN, as well as, the ventromedial and the dorsomedial hypothalamus.

1.7 C-Fos

C-Fos is a nuclear transcription protein produced by proto-oncogene, and is a useful marker for tracing neuronal activities in the brain after conditional stimuli of experimental animals. Proto-oncogenes function in several aspects of signal transduction processes.

Its' function is in the transmission of information between and within cells. In the majority of cell types, the basal level of c-Fos mRNA, and protein statements are relatively low. In these cell types, extracellular signals are required constantly to maintain elevated levels of statement. One of the features of c-Fos suggests that it might function in the signaling processes (Greenberg and Ziff, 1984, Kruijer et al. 1985; Muller et al., 1984), and it is now known that many types of stimuli elicit induction of c-Fos mRNA and protein.

C-Fos occurs within 15-20 minutes after transcriptional activation (Greenberg and Ziff, 1985; Greenberg et al., 1986). The mRNA accumulates and reaches peak values at 30-45 minutes post-stimulation (Muller et al., 1984). Thereafter, it declines with a relatively short half-life of about 12 hours. Synthesis of c-Fos protein follows mRNA expression and it is turned over with a half-life of about 1 day (Muller et al., 1984; Curran et al., 1984). The induction of c-Fos transcription occurs in the presence of protein synthesis inhibitors, thus suggesting that the proteins required for statement are present in unstimulated cells, and that they are activated

by post-translational modification. This feature led to the classification of Fos and other rapidly induced genes as cellular immediate-early genes by analogy to the immediate-early genes of viruses (Lau and Nathans , 1987; Curran and Morgan, 1987).

In the normal mouse brain, c-Fos staining mainly represents the distribution of a 35kDa, Fos-related antigen (Morgan and Curran., 1989). It is predominantly found in granule cells of the dentate gyrus, some pyramidal neurons within the hippocampus, neurons in the piriform and anterior cingulate cortices, the anterior olfactory nucleus, and in the bed nucleus of the stria terminalis, the amygdala, and sporadically found in the cerebral cortices (Dragunow M. et al, 1989).

2.1 Hypotheses and Aims

General Hypothesis: a high-fat diet may alter hypothalamic neuronal activity in hypothalamus which are critically involved in body energy balance.

Specific Hypothesis:

1. Changed neuronal activity in the hypothalamus can be revealed by using c-Fos as a marker, which indicates activation of neurons by the experimental conditions.
2. A high-fat diet may increase NPY contents in those hypothalamic areas critically involved in body energy balance.
3. A high-fat diet may increase alpha-MSH contents in those hypothalamic areas critically involved in body energy balance.
4. A high-fat diet may decrease leptin receptor mRNA statement in those hypothalamic areas critically involved in body energy balance.

General Aims: The general aim of this study is to examine hypothalamic neural activity influenced by high or low fat diet. The study will examine both short (1 week) and medium term (8 weeks) effects of the different diets. Since this is my one year research project, long term dietary effects were not examined here. Further, body weight gain, energy intake, and epididymal fat mass were also measured after high and low fat diets were administered.

Specific Aims:

Experiment 1: To examine hypothalamic c-Fos activity after eating high-fat diet for 1 and 8 weeks.

Experiment 2: To examine hypothalamic NPY activity after eating a high-fat diet for 1 and 8 weeks.

Experiment 3: To examine hypothalamic α-MSH activity after eating a high-fat diet for 1 and 8 weeks.

Experiment 4: To examine hypothalamic leptin receptor mRNA statement after eating a high-fat diet for 1 and 8 weeks.

Chapter 2: Methods

2.1 Mice and histology

2.1.1 Type of the mice used

C57BL/6J (+/+) mice were used in this study.

Although there are four types of mice used in studying obesity and diabetes (C57Bl/6J, C57Al/6J, Agouti, and Tubby), C57Bl/6J is the most suitable type to use to study diet-induced obesity because C57Bl/6J is prone to induce diet-induced obesity, and a diet-induced obese C57Bl/6J mouse model mimics human obesity. Twelve C57Bl/6J mice were used in this study. The mice were separated into two groups: one was fed a low-fat diet, and the other, a high-fat diet. The other three mice were sacrificed after eight weeks of eating a high, or low fat diet. It is noted that present studies can only provide preliminary data due to the low number of mice used. For this is one year research project, I plan to examine c-Fos, NPY, alpha-MSH and Leptin receptors on tissue sections. Processing histological sections and quantification are a time consuming task; therefore, I don't have enough time to do much more work than this.

2.1.2 Feeding procedure

The mice used in this study were three weeks old, male, and were purchased and transported from the Animal Resource Centre in Western Australia. After the mice arrived, they were housed in the Animal House at the University of Wollongong. These mice were housed individually in the standard cages (30cm x 15cm x 10cm).

The mice house was quiet, a 12hr/12hr light/dark cycle (lights on 7:00am) occurred every day and they were in a temperature-controlled environment (20°C). They were allowed ad lib access to water for the entirety of the study. For the first week, the mice were fed a standard laboratory chow.

At the end of their one week lab chow diet, the mice were weighed and then administered a high or low fat diet. Of the 12 mice, 6 mice were sacrificed after one week on a high- or low-fat diet. Another 6 mice were sacrificed after 8 weeks of A high- or low fat feeding. The amount of food eaten by each mouse was weighed every two days, and the body weights of the mice were weighed every week.

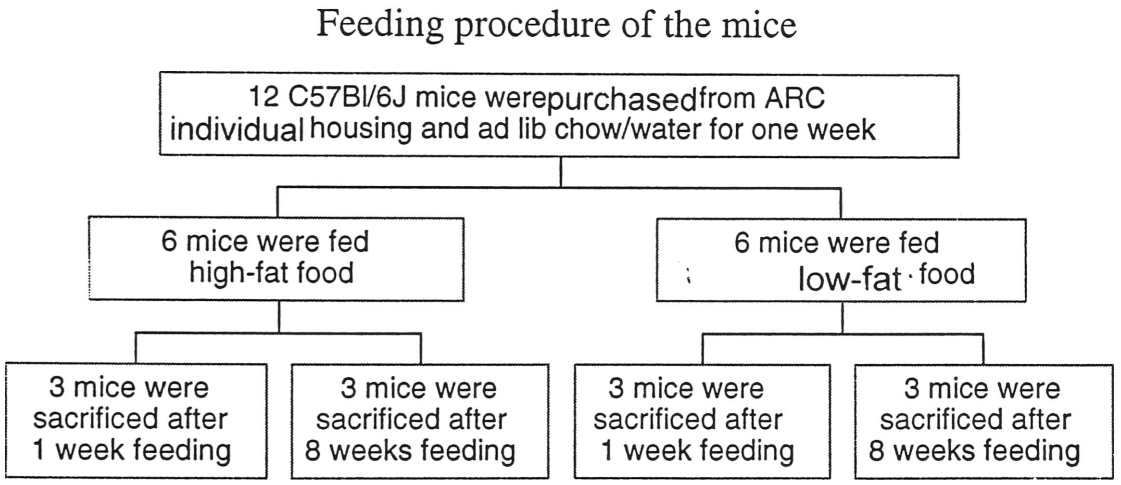

Figure 2.1: Feeding procedure for the mice.

In the high-fat diet group, fat accounts for about 58.7% of total energy. In the low-fat diet group, fat accounts for about 9.7% of total energy.

2.1.3 The diet of the mice

The composition of the Low-Fat and High-Fat diets is as follow:

Component	High-Fat: Gram (%)	Low-Fat: Gram (%)
Safflower oil	24.0	4.0
Tallow Stearin	8.0	
Casein	33.5	25.3
Sucrose	10.0	10.0
Starch	7.2	48.4
Vitamin mix	1.4	1.0
Mineral mix	9.8	6.9
Cellulose powder	5.6	4.0
DL-Methionine	0.5	0.4
Fat energy, kcal/100g(%)	288.0 (58.7)	36.0 (9.7)
Carbohydrate, kcal/100g(%)	68.8 (14.0)	233.6 (63.0)
Protein, kcal/100g(%)	134.0 (27.3)	101.2 (27.3)
Total energy, kcal/100g(%)	490.8 (100.0)	370.8 (100.0)

Table 2.1: Compositions of high-fat and low-fat diets.

Everyday, at 2:00pm, the food left on the plate was weighed and then fresh food was provided. The food intake for every mouse was calculated everyday.

The energy expenditure was calculated from the amount of food intake. The converting factors for calculating energy expenditures are as follows: 1 gram carbohydrate = 4 calories; 1 gram fat = 9 calories; 1 gram protein = 4 calories.

2.1.4 Histology

The mice were sacrificed by using an overdose of Nembutal 120mg/kg i.p. (0.7~0.9 ml). After an intracardiac perfusion through the left ventricle with saline (0.9% NaCl) for 5 minutes, the tissue was perfused by 4% paraformaldehyde in PB (phosphate buffer) for about 15 minutes, to fix the brain. Then the brains were dissected and removed from the skulls. Afterward, the mouse brain was immediately put into 4% paraformaldehyde and stored for 12 hours. The fixed brains were put into 30% sucrose with PB for about 20 hours until all the brains had sunk to the bottom of the bottles. Lastly, the brains were frozen in -70°C and wrapped with aluminum foil and stored in -70°C. The frozen mice brains were sectioned entirely by freeze microtome at 25μm thickness in –17°C. The sections were divided into four groups. The first three sets were used for C-fos, α-MSH and NPY respectively, and the fourth set was used for the detection of of leptin receptor mRNA.

2.2 Immunohistochemistry

2.2.1 Theoretical knowledge

Immunohistochemistry relies upon the specific binding of an antibody to the antigen. The primary antibodies used in this study were C-Fos, NPY and alpha-MSH. All these primary antibodies were derived from rabbit. The secondary antibody used in this study was anti-rabbit IgG conjugated to biotin.

In order to obtain the colour on the detected antigen-antibody complex, extravidin was used. Avidin is a basic glycoprotein (mol. wt. 68kDa), which has a

high affinity for the biotin. The avidin-biotin reaction in these detections is Extravidin-Secondary Antibody binding. Immunocytochemical staining methods for light microscopy depend on enzyme-substrate reactions, these sections convert colourless chromogens into coloured and visible end product, a known end product, which is insoluble in alcohol and xylene. Diaminobenzidene (DAB) is the enzyme-substrate that was used in this experiment.

2.2.2 Procedure

For immunohistochemistry the sections were firstly washed in 50% alcohol and 30% H_2O_2 mixture for 30 minutes. Blocking solution is made from 0.1M phosphate buffer (pH=7.4) and Normal Sheep Serum (NSS). The primary antibody (C-Fos, α-MSH, or NPY) was diluted at 1:8,000 (especially for NPY, dilution is 1:16,000) in phosphate-buffered Saline (PBS) containing 0.1% Triton x100 (PBS-Triton). They were then incubated for four days at 4°C.

After three-10 minute washes in PBS, the sections were incubated in secondary antibody diluted with PBS-Triton for 2-4 hours. The sections were washed in PB for three times 10-minute at 1:100 in Tris-HCl. Then they were incubated in Extravidin diluted at 1:1000 PBS-Triton for 2.5 hours. The sections were incubated in DAB MIX containing 10mg/ml DAB, 0.4% Amc, 20% D-Glucose and 4% NAS for 10 minutes.

Finally, the sections were put into DAB MIX and Glucose Oxidase mixture (DAB MIX : Glucose Oxidase = 100:1) for 5~7 minutes. The colour was checked

for staining under the microscope. The sections were washed in Tris-HCl for three times for 10-minute, and then mounted on slides coated with gelatin.

2.3 In Situ hybridisation

2.3.1 Theoretical knowledge

In the detection of the leptin receptor, I used the labelled oligonucleutide probe whose DNA contains complementary sequences to hybridised to the cellular LR mRNA on the mice brains' sections. The technique of In Situ hybridisation consists of five principal steps: tissue preparations, oligonucleutide labelling, hybridisation, washing and detection.

2.3.2 Procedures

Probes: In this study, there are two oligonucleotide probes used. Both probes are designed for the conserve region of leptin receptor. The sequences of these two antisense leptin receptor probes are:

5'-CCGATTTCCCACATCTTCTGACCACCACGTCCCATTGTGGGCA-3' and

5'GCTAACTGGGTCACTCACAATGCTGTACTGTATCTCAGGGA-3'.

No significant homology to the designed probes was found in the Gene Bank. One control probe combined by two oligonucleotide probes of Tubby is also used: Tub1.1 sequence(5' to 3'): ATC CAG TCG GAA TGC GGC TTG GAA GTC ATG TCT CCC GGG GGC GGG and Tub1.2 sequence(5' to 3'): GCC CAT CAG GTT GGA CCG CAA TTT CCC GAT ATA GCT ATC GCC TCC CCG

I used Tubby as a positive control due to its extensive expression in brain tissue. All three probes listed above were tailed with DIG at the 3' end using terminal transferase (Boehringer Mannheim, Germany). The labelling efficiency was verified with the spot test (DIG User's Guide Manual, Boehringer Mannheim, 1995).

Tissue hybridization and detection: The sections were warmed from -70°C to room temperature prior to pretreatment as described previously. Briefly, the sections were washed twice in saline sodium citrate (SSC), and then treated with 2.5 (g/ml) proteinase K for 8 min at 37°C. To stop the protein digestion, sections were washed with 0.2% glycine. Thereafter the sections were placed initially into a prehybridization solution containing Denhardt's solution, 50% formamide, 250μg ml-1 salmon sperm DNA, 250 g ml-1 yeast t-RNA, and 10% dextran sulphate and incubated at 38°C in a hybridization solution containing 1.2 pmol ml-1 DIG-labelled probe for 4h. The sections were washed three times in 2xSSC at room temperature, 39°C and room temperature for 15 min each, respectively. Following the washes, I used the standard colorimetric detection system with NBT and X-phosphate as substrate to detect the labelled signals (Boehringer Mannheim, Germany).

Controls: In order to clarify signal specificity, two controls were used: the hybridization buffers with Tubby probe and no probe.

Chapter 3 : C-Fos immunoreactivity

3.1 Experiment Design

1.Assignation of treatment groups:

Twelve, 3 weeks old, C57BL/6J mice were obtained from the Animal Resources Centre and fed a standard laboratory chow diet for 1 week to accommodate to the new environment. Afterwards, they were separated into four groups: the mice fed with a high-fat diet for 1 week and 8 weeks, the mice fed with a low-fat diet for 1 week and 8 weeks.

Aim: To compare c-Fos-like immunoreacitivity in the hypothalamus of the mice fed with a high-fat and a low-fat diet for 1 and 8 weeks.

2. Comparisons:

Group 1 vs group 2: the mice fed with a low-fat diet for 1 week versus the mice fed with a high-fat diet for 1 week.

Group 3 vs group 4: the mice fed with low-fat diet for 8 weeks versus the mice fed with high-fat diet for 8 weeks

3.2 Materials

1. Histology

The mice were sacrificed by an overdose of abdominal injection of Nembutal (120mg/kg). The mice were perfused with 0.9% NaCl 100ml and followed by 4% Paraformaldehyde 200ml. Brains were dissected out and stored in 4% Paraformaldehyde for 12 hours for post fixation. Brains tissues were then put into 30% sucrose for about 20 hours. They were then cut at a 25μm thickness by the cryostat machine in –17°C.

2. Antibody Dilution

In order to get the maximum contrast between positively staining and background, the concentration of primary antibody for c-Fos was tested for the best concentration of 1:8,000.

3.3 Methods

1. Negative control

Brain sections subject to immunostaining procedures in the absence of the primary antibody did not show any signals.

2. Measure the body weight, food intake and fat mass.

I measured the body weight and food intake of the mice since their ad lib chow feeding had begun, until they were 8 weeks into the dietary feeding. The epididymal fat mass weights were measured before their sacrificing.

3. Data analysis:

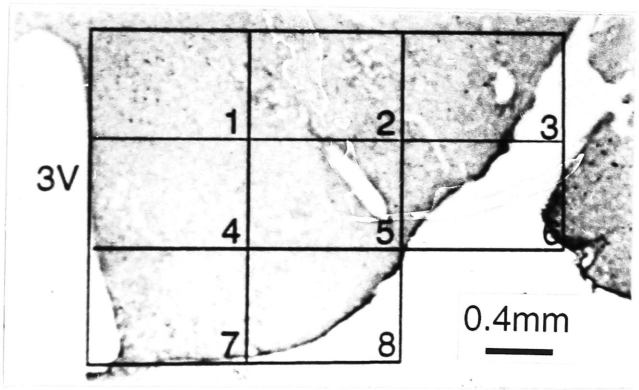

Figure 3.1: Photograph of mouse brain section taken at 5X magnification showing positively immunostained neurons. Squares appear in the photo are for results analysis.

In order to quantify the amount of immunoreactivity of c-Fos-like immunoreactive neurons, the hypothalamus was divided into eight squares when counting the FLI positive staining in the square. From the picture, it is shown there are eight squares on each side numbered from 1 to 8. For example, the DMH area is in square 1 and in two third of square 2 (Keith B. J. Franklin, George Paxinos, 1997). The VMH is found in most part of the square 4, and in half of the part of square

7, half of the part of square 8 and in the one sixth of the part of square 5. LH is in square 3 and almost takes half of the part of square 6. To find PeF under the microscope of 40x10 magnification, it can be seen that its' shape looks like a blood vessel. After finding all the nucleus in the correct locations, the stained c-Fos cells were counted.

In the cell counting, background staining remained low, and C-fos positive immunoreactivity cell bodies were easily detected under the microscope. Although a few lightly stained cell bodies were difficult to be detected since the colour of staining change variously from dark to shallow brown, I only chose dark and deep brown colour staining as the FLI positive staining to count. Results for each sub-experiment will be displayed separately.

3.4 Results

3.4.1 Body Weight

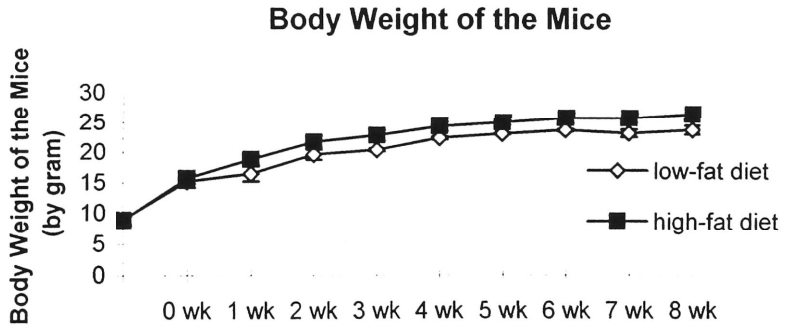

Figure 3.2: Body weight of the mice. Data shown as mean ± SE for groups of six mice.

Obesity was induced in three week old C57Bl/6J mice by exposure to a diet in which 58.7% of calories were derived from fat; the comparison group of mice were fed a 9.7% fat diet. During the one week of ad lib chow feeding, both high-fat and low-fat dietary mice increased their body weight in parallel. Also both of groups increased their body weight gradually during the 8 weeks of different diet feeding. After the first week of different diet feeding, the body weight of HF

fed mice were more than that of LF fed mice and eventually there was an obvious 2.7 gram difference between them. HF fed mice were 7.3% heavier than LF fed mice after 8 weeks of different diet feeding.

3.4.2 Food intake

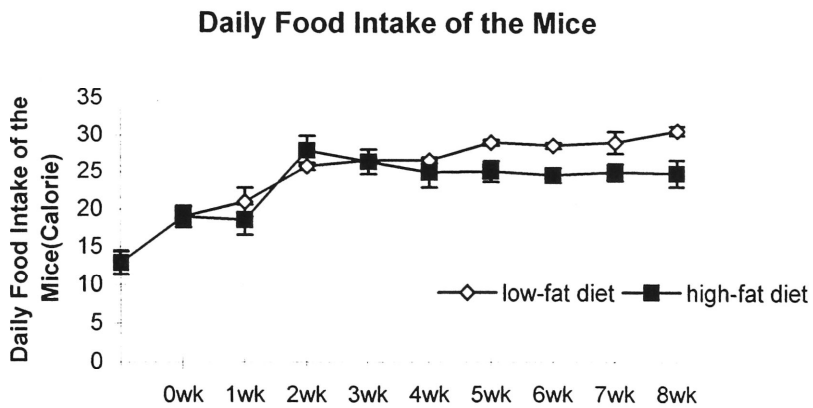

Figure 3.3: Food intake of the mice. Food consumption (by calorie) in C57Bl/6J mice fed a high-fat diet or a low-fat diet during the development of dietary induced obesity. Data shown as mean±SE for groups of six mice.

There were three stages for the food intake of HF fed mice. In the first week of feeding, the food intake was decreased and then increased in the second week. After the second week of feeding, the food intake of the HF fed mice decreased a little then remained almost at the same level which was lower than that of LF fed mice during the next feeding. The food intake of LF fed mice was increased gradually unlike the three stages the high-fat diet mice were fed. Eventually, their food intake changes were 3.67 calories different. Food intake of the LF-fed mice was found to be 23.2% more than that of the HF-fed mice.

3.4.3 Epididymal fat weight

Feeding period	1 week (n=6)		8 weeks (n=6)	
Diet	HF	LF	HF	LF
Epididymal fat mass (mg)	213±2.97	203±7.19	570±2.05#	369+2.78

Table 3.1: Epididymal fat weight in the different feeding periods of the mice after its' high-fat or low-fat diet. Results are the mean±SE for groups of 6 mice. # P<0.0001; high-fat diet fed mice (HF) vs low-fat diet fed mice (LF) by Fisher's protected least-significant difference test.

3.4.4 Fos-like Immunoreactivity results:

Areas	LF fed mice		HF fed mice	
Hypothalamus	1 weeks (n=6)	8 weeks (n=6)	1 weeks (n=6)	8 weeks (n=6)
Arc*#	1.8±0.25	0.8±0.31	3.5±0.43	2.3±021
VMH*#	2.5±0.43	3±0.52	2.2±0.31	8.7±0.56
LH*#	0±0.0	5.5±0.76	8.2±0.60	14±0.58
DMH*#	5.2 ±0.48	12.2± 0.48	16.2±0.31	20.2±0.60
PeF	0±0.0	0±0.0	0±0.0	1±0.37
PVN	0.8±0.31	1.2±0.31	1.2±0.31	2.6±0.33

Table3.2 Distribution of C-fos-like immunoreactive neurons in hypothalamus and low-fat dietary mice at different times: after 1 week and 8 weeks dietary feeding.

means ± S.E.: *p=0.05, or less for age effect where values in a given area from the mice fed for 1 week compared with those fed for 8 weeks by two-way ANOVA; #p=0.05, or less for diet effect where values in a given area from HC fed mice were compared to those from HF fed mice by two-way ANOVA.

PVN: paraventricular hypothalalmic nucleus; Arc: arcuate hypothalamic nucleurs; VMH: ventromedial hypothalamic nucleus; LH: lateral hypothalamus; DMH: dorsomedial hypothalamic nucleus; PeF: perifornical nucleus

Group 1 vs group 2: the comparison was made in mice fed with a low-fat diet, or a high-fat for 1 week.

P-values obtained for t-test comparison of number of FLI neurons counted in hypothalamic regions of C57Bl/6J mouse brains.

Region	Arc	VMH	LH	DMH	PeF	PVN
P-value	0.0101	0.5413	<0.0001	<0.0001	_	0.0023

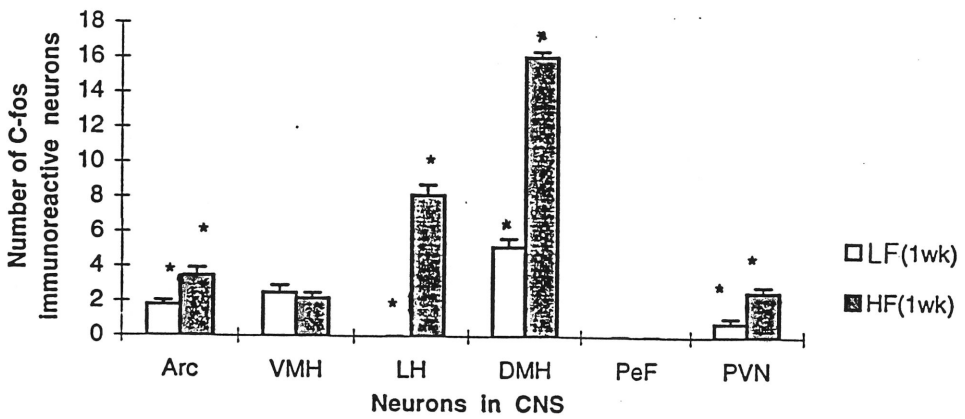

Figure 3.4: comparison of the number of FLI neurons in hypothalamic regions of the mice fed with the low-fat diet for 1 week vs the mice fed with the high-fat diet for 1 week. (Mean±SE, * denotes significant differences ($p<0.05$))

In Arc, the number of the FLI positive staining of the mice fed with a high-fat diet for 1 week was significantly (49%) greater than that of the mice fed with a low-fat diet for 1 week ($p<0.05$).

In LH, the number of the FLI positive staining of the mice fed with a high-fat diet for 1 week was significantly (100%) greater than that of the mice fed with a low-fat for 1 week ($p<0.05$).

In DMH, the number of the FLI positive staining of the mice fed with a high-fat diet for 1 week was significantly (68%) greater than that of the mice fed with a low-fat diet for 1 week ($p<0.05$).

In PVN, the number of the FLI positive staining of the mice fed with a high-fat diet for 1 week was significantly (33%) greater than that of the mice fed with a low-fat diet for 1 week ($p<0.05$).

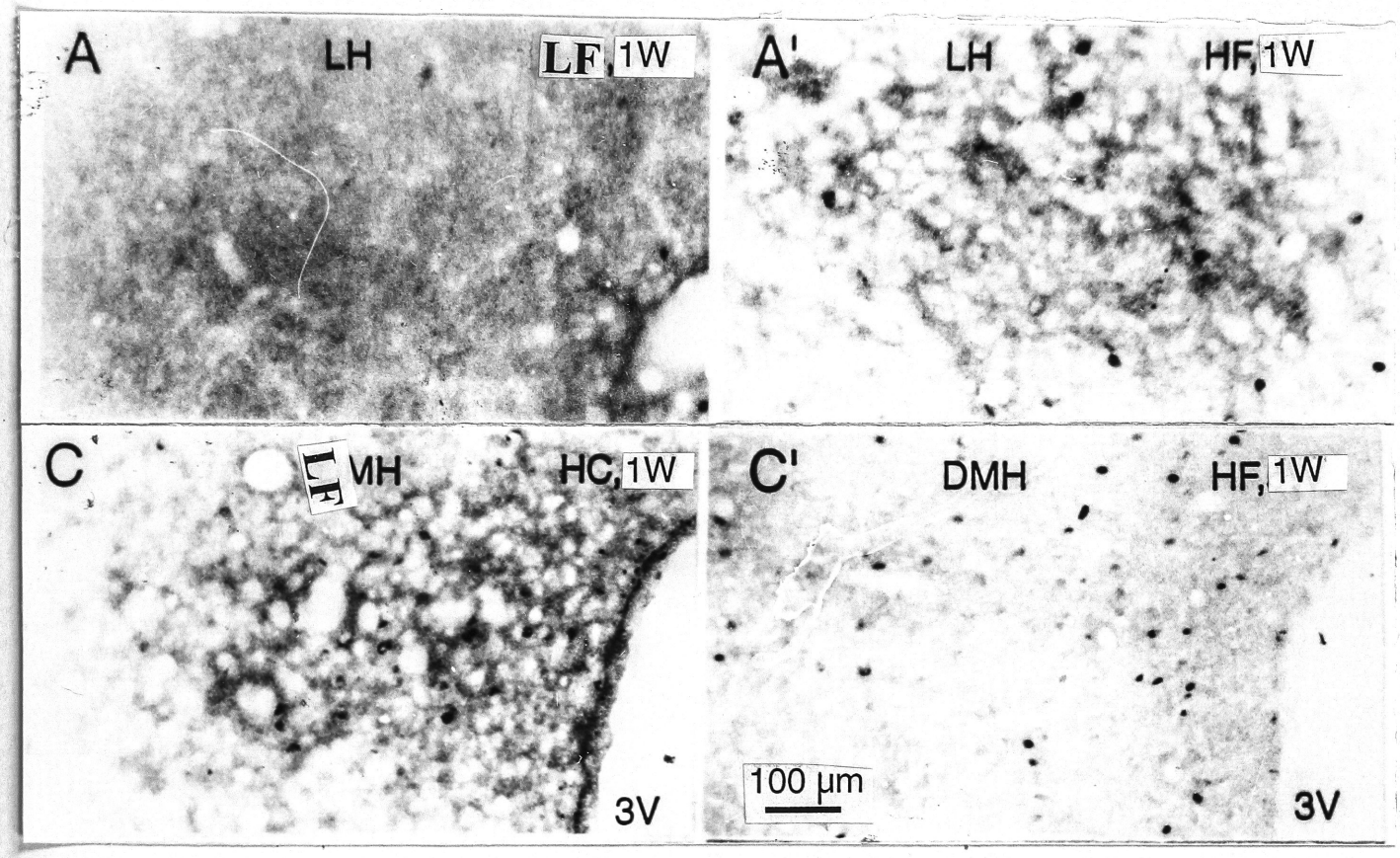
A
LH
LF, 1W
A'
LH
HF, 1W
C
LF
MH
HC, 1W
3V
C'
DMH
HF, 1W
100 µm
3V

Figure 3.5: Photomicrographs (A, A', C, C') of 25μm coronal sections of the C57Bl/6J mouse brain, immunohistochemistry stained for the presence of C-fos. A, C are from the mice fed with a low-fat diet for 1 week, and A', C' are from the mice fed with a high-fat diet for 1 week. All sections are approximately at the level of bregma (-1.58) and photographs were taken at 20X magnification. Pairs of sections (A, A', C, C') show increased numbers of positively stained neurons in the mice fed with high-fat for 1 week compared to the mice fed with low-fat for 1 week. (A, A') lateral hypothalamic area (LH), (C, C') dorsomedial hypothalamic nucleus (DMH).

Group 3 vs group 4: the comparison was made in mice fed with a low-fat diet and a high-fat diet for 8 weeks.

P-values were presented in the following table for the number of FLI neurons counted in hypothalamic regions of the mouse hypothalamus.

Region	Arc	VMH	LH	DMH	PeF	PVN
P-value	0.0024	<0.0001	<0.0001	<0.0001	0.0209	1.0

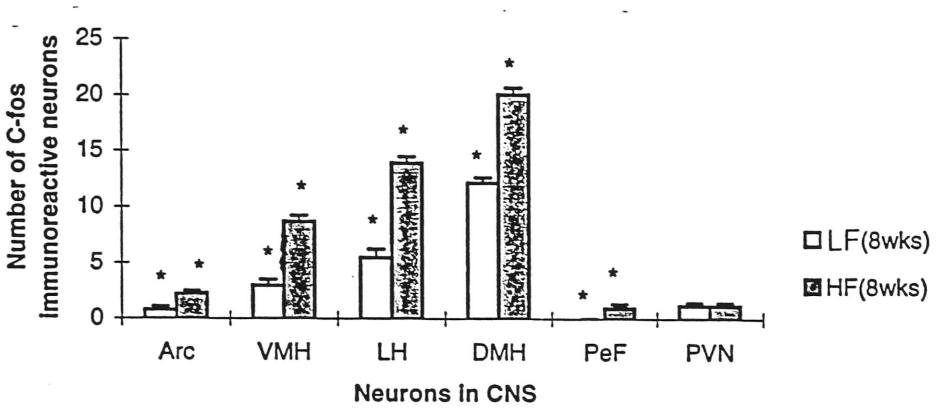

Figure 3.6 compares the number of FLI neurons in hypothalamic regions of the mice fed with a low-fat diet for 8 weeks and the mice fed with a high-fat diet for 8 weeks (Mean±SE, * denotes significant differences ($p<0.05$)).

In Arc, the number of the FLI positive staining of the mice fed with a high-fat diet for 8 weeks was significantly (57%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In VMH, the number of the FLI positive staining of the mice fed with a high-fat diet for 8 weeks was significantly (66%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In LH, the number of the FLI positive staining of the mice fed with a high-fat diet for 8 weeks was significantly (61%) greater than that of the mice fed a low-fat diet for 8 weeks ($p<0.05$).

In DMH, the number of the FLI positive staining of the mice fed with a high-fat diet for 8 weeks was significantly (40%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In PeF, the number of the FLI positive staining of the mice fed with a high-fat diet for 8 weeks was significantly (100%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

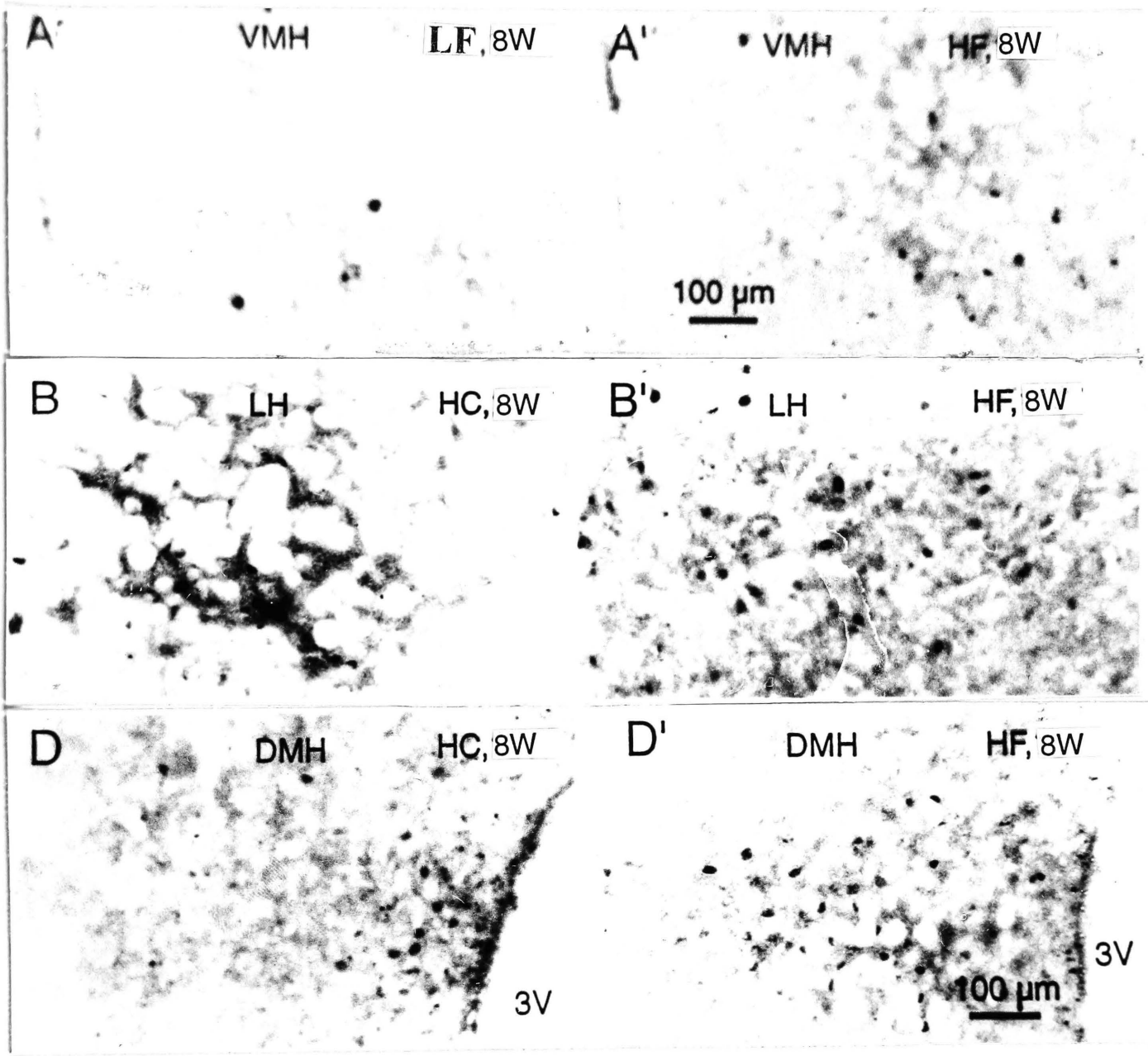
A
VMH
LF, 8W
A'
VMH
HF, 8W
100 μm
B
LH
HC, 8W
B'
LH
HF, 8W
D
DMH
HC, 8W
3V
D'
DMH
HF, 8W
3V
100 μm

Figure 3.7: Photomicrographs (A, A', B, B', D, D') of 25μm coronal sections of the C57Bl/6J mouse brain, which were immunohistochemical stained for the presence of c-Fos. A, B, D are from the mice fed with low-fat for 8 weeks and A', B', D' are from the mice fed with high-fat for 8 weeks. All sections are approximately at the level of bregma (-1.58) and the photographs were taken at 20X magnification. Pairs of sections (A, A', B, B', D, D') show increased numbers of positively stained neurons in the mice fed with high-fat for 1 week compared to the mice fed with low-fat for 8 weeks. (A, A') ventromedial hypothalamic area (VMH), (B, B') lateral hypothalamus (LH), (D, D') dorsomedial hypothalamic nucleus (DMH).

3.5 Discussion

This study is only part of large project carried out in Dr. Huang's laboratory. It has been shown, just after 7 weeks of the feeding, that the C57Bl/6J mice have started to dramatically increase their body weight, and that there is a difference in body weight between the high fat and the low fat groups (Lin and Huang, 2000). Similar reports are seen by Surwit et al (Richard R.S., 1988). This significant separation of body weight between the two occurs at about 10 weeks of feeding. This study is to show the hypothalamic neuronal changes prior to the dramatic increase in body weight.

The food composition for diet-induced obese mice and control mice are different: The group of diet-induced obese mice was given a high-fat diet containing 58.7% fat, 14% carbohydrate and 27.3% protein. The controls group was given a low-fat diet containing 9.7% fat, 63.0% carbohydrate and 27.3% protein. In order to provide close similarity of dietary composition between these diets, the low fat diet was freshly made at the same time as the other. The normal laboratory chow food was not used in this study because it is difficult to know its precise food composition and the condition of the food oxidation.

The hypothalamu plays a very important role in body weight control. It receives the information from the peripheral, especially from the fatty tissue (Levin B.E., 1983). The hypothalamu receives the signals from leptin which is a major signal that indicates the body fat mass and activates the brain autonomic nervous system to regulate body weight. This pathway is called the neural pathway regulation of body weight. This occurs mainly through the hypothalamic, the brainstem's nucleus and the efferent pathway. On the other hand, the hypothalamus can activate neural endocrine pathways which act through the hypothalamic pituitary adrendual pathway. The nuclei of its pathway in the body weight control are Arc, LH, PVN, VMH and DMH. (Huang et al, 1998). In this

discussion, I will mainly focus on the hypothalamic c-Fos immmunoreactivity in the mice fed with a HF diet for 1 week and for 8 weeks. I will first describe the Fos immunoreactivity in the hypothalamus after 1 week on a HF diet. I will then describe the c-Fos immunoreactivity in the mice fed with a HF diet for 8 weeks. Finally, I will compare the two groups. Then, I will try to explain the possible involvement of the time factor.

C-Fos is a product of proto-oncogene called the neuclear transcription protein. It is commonly used as the marker to detect the activated neurons on the stimuli. The C-Fos gene expression can be induced after 20 minutes of stimulation, and c-Fos protein can be visualised by using immunohistochemistry after 30 minutes of condition stimuli. This activated c-Fos expression will last for over three days. In my study, the mice were fed with a HF diet ad libtum. The first group was fed for one week. The second group was continually fed with a HF diet for 8 weeks. The control group was continually fed ad libitum with a LF diet. Fos immunoreactivity in the brain was compared among the four groups. Although the c-Fos detection is a very reliable technique and has been used widely, the disadvantage of detecting the Fos immunoreactivity is that it can not tell the specific transmitters activity. It is a rather robust method to indicate the overall neural activity rather than stimuli. Therefore, the control procedure is very important. The mice should not be disturbed a half hour prior to sacrificing. Further, there is a fluctuation of c-Fos activity during the day. Therefore, in this experiment, the sacrificing time was noted to be in the early morning between 4:00 AM and 6:00 AM. All of the mice were in satiated status.

Previous studies from these groups have demonstrated that c-Fos immunohistochemistry can be used to determine the brain areas that are involved in the regulation of energy balance (Huang X.F., 1998; Lin S., 1999; Wang H., 1999).

After the mice were fed a HF diet for one week, the c-Fos immunoreactivity significantly increased in LH, DMH and Arc. There were no differences in VMH between HF diet and LF diet. The large increase in neural activity found in this study might be in part responsible for the increased food intake after one week on a high-fat diet (Figure 3.3).

It was clearly shown that the LH had the largest (100%) increased c-Fos immunoreactivity on the HF diet. The lateral hypothalamus is known as a feeding center and activation is associated with increased adiposity (Bray G. A., 1981). Neurons in the LH project to autonomic regulatory centres in the brainstem, such as the dorsal motor nucleus of vagus, solitary nucleus, lateral parabrachial nucleus, and the lateroventral periaqueductal gray (Zardetto Smith A.M. et al, 1988). The LH contains neurontransmitters, such as melanin-concentrating hormone, orexin, and neuropeptide Y (NPY), which promote adiposity (Meister B. et al, 1998; Shimada M. et al, 1998; Stanley B.G. et al, 1985; Wilding J.P. et al, 1992; Williams G. et al, 1988; Zardetto Smith A. M. et al, 1988). Further, previous studies showed that high-fat diet-induced obese mice had low sympathetic nervous system activity and metabolic rate, and high parasympathetic activity (Matsuo T. et al, 1995). Stimulation of the LH increased food intake, body adiposity, and parasympathetic activity (Friedman J.M., 1997; Leibowitz S.F., 1995). On the other hand, lesions to the LH result in an increased sympathetic activity accompanied by decreased food intake, lower adiposity, increase oxygen consumption, and core temperature; the net result is a maintenance of reduced body weight (Bernardis L.L. et al, 1993; Bray G.A. et al, 1981; Keesey R.E. et al, 1997). This study showed that the DIO mice significantly increased FLI neurons in the LH compared to the low-fat diet mice. It suggested that the increased neuronal activity in the LH contributes to the decrease in sympathetic activity, energy expenditure, and metabolic rate, thus promoting the development of diet-induced obesity.

It has also been found that there is a large increase in the number of C-fos neurons in the DMH. It has been known that DMH has rich connections with the vagus complex in the brainstem. It plays an important role in body weight regulation with some potential mechanisms described (Bernardis L.L. et al, 1998). For example, microinjection of excitatory amino acid into the DMH resulted in a decrease of sympathetic nerve activity supplying brown adiposity tissue (Yoshimatsu H. et al, 1993), and NPY mRNA expression was increased in the DMH of DIO mice (Guan S. M. et al, 1998). This indicates that DMH lesions offer some protection against high-fat diet-induced obesity. In this study, it does indicate that the DMH is one of most sensitive areas in the brain in response to a HF diet, which is a strong stimulus-inducing food intake.

The significant increase of c-Fos activity in the Arc nucleus might contribute to the changes of increased food intake (Figure 3.3). It was well known that Arc played an important role in body weight control, including food intake and energy balance (Satoh N. et al, 1997). Blood-born leptin could activate leptin receptors of neuropetide Y (NPY) neurons in the Arc. The negative feedback regulated by leptin on Arc NPY production was also reported (Wang Q. et al, 1997). This study showed that mice, after a high-fat diet feeding for one week, had increased food intake accompanied by increased c-Fos immunoreactivity in the Arc. Thus, it is possible that those activated FLI neurons are NPY-containing neurons.

After the mice were fed with HF diet for eight weeks, the most striking changes in c-Fos immunoreactivity were found in the DMH. The LH was also

found to have an increase in c-Fos immunoreactivity. As I described previously, it contributed to the high-fat diet feeding.

In this study, we have found that the VMH showed a large increase in c-Fos immunoreactivity. This indicates many neurons were activated after eight weeks on a high-fat diet. The function of VMH is opposite to that of LH, therefore in this study, it might contribute to the decreased calorie intake which is found after eight weeks on a HF diet (Figure 3.3).

As I discussed above, the HF diet can induce c-Fos immunoreactivity in both 1 week and 8 weeks on a HF diet. Here, I would like to question if it is different in proportion to a HF and LF group. As can be seen in Table 3.3, the nucleus including the Arc, DMH and LH showed a decrease in percentage of increased Fos immunoreactivity, when comparing the HF diet group to a LF diet group. However, the only nuclei VMH, showed that there was an increase in percentage of the already increased Fos immunoreactivity, which is almost 15 times more when comparing the HF diet group to the LF diet group.

There were a 20% increase in Fos immunoreactive neurons in VMH after a LF diet for a length of 8 weeks. However, the VMH increased the c-Fos immunoreactivity by up to almost 3 times when on a HF diet for 8 weeks. Therefore, the proportion of increased c-Fos immunoreactivity in a HF diet group is much greater than that of a LF diet group (15 times). The significance of this largely increased immunoreactivity in a HF diet group supports the results obtained in this study. It indicates that the overall calorie intake was decreased, and this supports that VMH is a satiety centre.

Hypothalamus	LF=(LF8-LF1)/LF1 (%)	HF=(HF8-HF1)/HF1 (%)	HF/LF
Arc	50	34	0.68
VMH	20	295	14.8
LH	450	75	0.16
DMH	135	20	0.15

Table 3.3: The increased percentage of increased FLI fed a low-fat or a high-fat diet for 8 weeks. Abbreviations: LF1: the number of FLI of the mice fed a low-fat diet for 1 week; LF8: the number of FLI of the mice fed a low-fat diet for 8 weeks; HF1: the number of FLI of the mice fed a high-fat diet for 1 week; HF8: the number of FLI of the mice fed a high-fat diet for 8 weeks.

Chapter 4: NPY immunoreactivity

4.1 Experiment Design

1.Assignation of treatment groups

Twelve, 3 weeks old, C57BL/6J mice were obtained from the Animal Resources Center and fed a standard laboratory chow diet for 1 week to accommodate to the new environment. Afterwards, they were separated into four groups: the mice fed with a high-fat diet for 1 week and 8 weeks, the mice fed with a low-fat diet for 1 week and 8 weeks.

Aim: To compare NPY immunoreacitivity in the hypothalamus in the mice fed with high-fat and low-fat diets for 1 and 8 weeks.

2. Comparisons:

Group 1 vs group 2: the mice fed with a low-fat diet for 1 week versus mice fed with a high-fat diet for 1 week.

Group 3 vs group 4: mice fed with a low-fat diet for 8 weeks versus mice fed with a high-fat diet for 8 weeks.

4.2 Materials

1. Histology

The mice were sacrificed by an overdose of abdominal injection of Nembutal (120mg/kg). The mice were perfused with 0.9% NaCl 100ml and followed by 4% Paraformaldehyde 200ml. Brains were dissected out and stored in 4% Paraformaldehyde

for 12 hours for post fixation. Brains tissues were then put into 30% sucrose for about 20 hours. They were then cut at a 25μm thickness by the cryostat machine in –17°C.

2. Antibody Dilution

In order to get the maximum contrast between positively staining and background, the concentration of primary antibody for NPY was tested for the best concentration of 1:16,000.

4.3 Methods

1. Detection of NPY Immunoreactivity

Immunohistochemistry technique was used in this study.

2. Negative control

Brain sections subject to immunostaining procedures in the absence of the primary antibody did not show any signal.

4.4 Results

Positive immunostaining for NPY was detected in all of the hypothalamic regions examined. Furthermore, under the microscope, background staining remained low and NPY positive immunoreactivity cell bodies were easily detected in the motor cortex.

In the present study, the quantification of NPY like immunoreactivity was carried out only in the hypothalamus. Positively stained buttons and neurons were counted. The colours after stained for immunohistochemistry against individual antibodies appeared to

be light brown, deep brown , and black. Only the black stained positively buttons were counted in this study.

Areas	LF fed mice		HF fed mice	
Hypothalamus	1 week (n=6)	8 weeks (n=6)	1 week (n=6)	8 weeks (n=6)
Arc*	990±25.9	1063±27.20	1005.8±57.7	1140±56.4
VMH*#	500.2±17.9	623±80.3	520±25.5	1428±48.2
LH*#	664.7±39.3	951±57.0	689±35.8	1556±31.7
DMH*#	925±46.1	1312.3±127.1	1059±188.6	1807±170.0
PeF#	370±19.1	300±13.4	489±29.5	490±16.1
PVN*#	1573.2± 61.2	1565.8±28.3	1626.5±13.2	1948.8±29.3

Table 4.1: Distribution of NPY immunoreactive varicosities in the hypothalamus of mice fed a low-fat diet were examined after 1 week and 8 weeks of feeding.

Means ± S.E.: *p=0.05 or less for the age effect where values in a given area from the mice which were fed with a different diet for 1 week which were compared to those which were fed with a different diet for 8 weeks by two-way ANOVA; #p=0.05 or less for diet effect where values in a given area from LF fed mice were compared to those from HF fed mice by two-way ANOVA .

PVN: paraventricular hypothalamic nucleus; Arc: arcuate hypothalamic nucleus; VMH: ventromedial hypothalamic nucleus; LH: lateral hypothalamus; DMH: dorsomedial hypothalamic nucleus; PeF: perifornical nucleus.

Group 1 vs group 2: The comparison was made in mice fed with a low-fat diet for 1 week vs the mice fed with a high-fat diet for 1 week.

The P-values were presented in the following table for the number of NPY-immunoreactive varicosities in hypothalamic regions of the mice hypothalamus.

Region	Arc	VMH	LH	DMH	PeF	PVN
P value	0.8074	0.5381	0.5079	0.6405	0.0067	0.4140

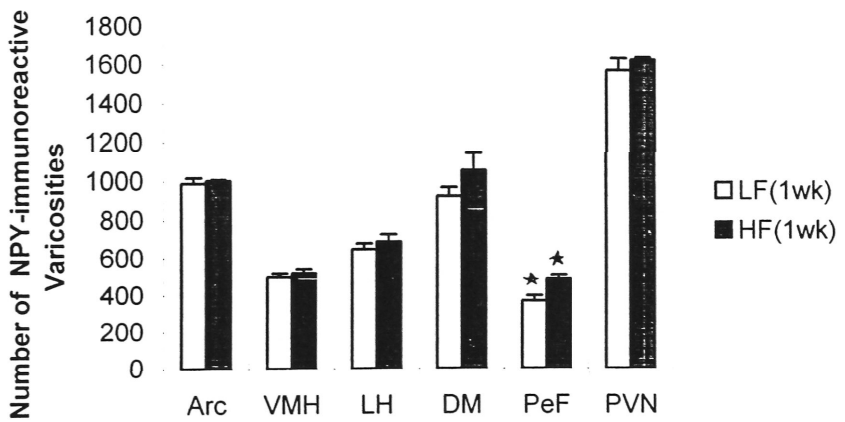

Figure 4.1: Comparison of a number of NPY-immunoreactive varicosities in hypothalamic regions of the mice fed with a low-fat diet or a high-fat diet for 1 week. (Mean±SE, * denotes significant differences ($p<0.05$))

In PeF, the number of the NPY positive staining of the mice fed with a high-fat for 1 week was significantly (24%) greater than that of the mice fed with a low-fat for 1 week ($p<0.05$).

Group 3 vs group 4: the comparision was made between mice fed with a low-fat diet or a high-fat diet for 8 weeks.

P-values were presented in the following table for the number of NPY-immunoreactive varicosities counted in hypothalamic regions of the mice hypothalamus.

Region	Arc	VMH	LH	DMH	PeF	PVN
P-value	0.2452	<0.0001	<0.0001	0.0417	<0.0001	<0.0001

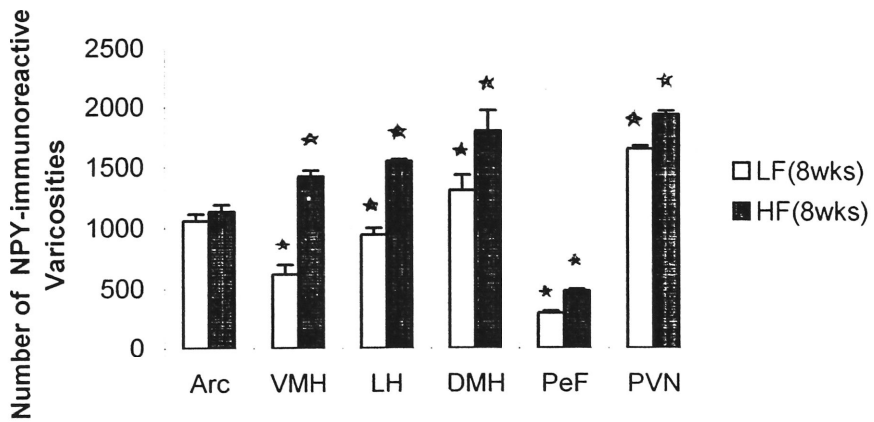

Figure 4.2: Comparison of a number of NPY positive boutons in hypothalamic regions of the mice fed with low-fat diet for 8 weeks vs the mice fed high-fat diet for 8 weeks. (Mean±SE, * denotes significant differences ($p<0.05$))

In VMH, the number of the NPY-immunoreactive varicosities of the mice fed with a high-fat diet for 8 weeks was significantly (98%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In DMH, the number of the NPY-immunoreactive of the mice fed with a high-fat diet for 8 weeks was significantly (27%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In LH, the number of the NPY-immunoreactive of the mice fed with a high-fat diet for 8 weeks was significantly (39%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In PeF, the number of the NPY-immunoreactive of the mice fed with a high-fat diet for 8 weeks was significantly (39%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In PVN, the number of the NPY-immunoreactive of the mice fed with a high-fat diet for 8 weeks was significantly (20%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

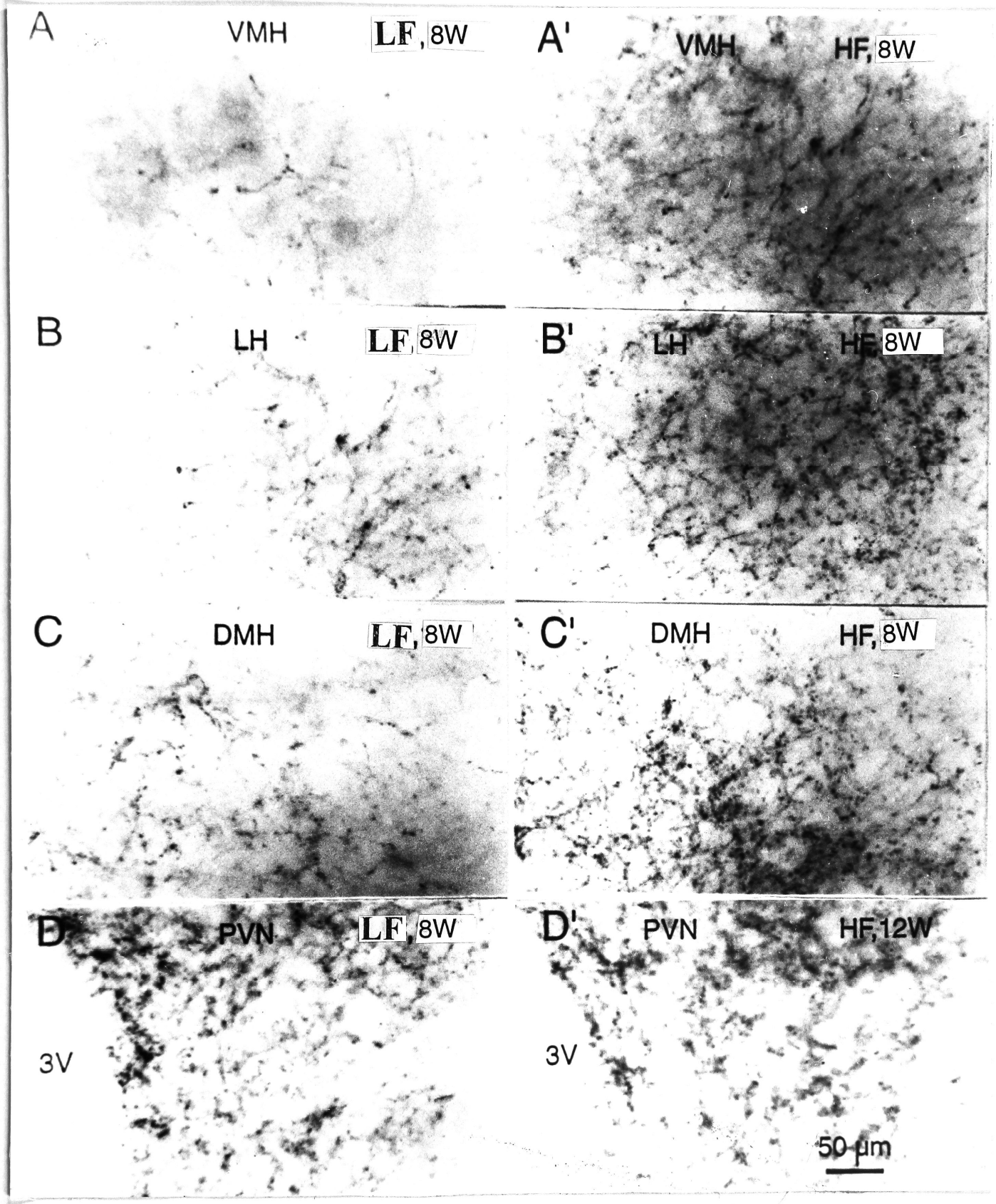
A
VMH
LF, 8W
A'
VMH
HF, 8W
B
LH
LF, 8W
B'
LH
HF, 8W
C
DMH
LF, 8W
C'
DMH
HF, 8W
D
PVN
LF, 8W
3V
D'
PVN
HF, 12W
3V
50 μm

Figure 4.3: Photomicrographs (A, A', B, B', C, C', D, D') of 25 μm coronal sections C57Bl/6J mouse brain immunohistochemical stained for the presence of NPY. A-D are from the mice fed with low-fat for 8 weeks and A'-D' are from the mice fed with high-fat for 8 weeks. All sections are approximately at the level of bregma (-1.58) and photographs taken at 40X magnification. Pairs of sections (A, A', B, B', C, C', D, D') show increased numbers of positively stained varicosities in the mice fed with high-fat for 8 weeks compared to the mice fed with low-fat for 8 weeks. (A, A') ventromedial hypothalamic area (VMH), (B, B') lateral hypothalamic area (LH), (C, C') dorsomedial hypothalamic nucleu (DMH), (D,D') paraventricular hypothalamic nucleu (PVN).

4.5 Discussion

Neuropeptide Y (NPY) is a physiological mediator of feeding. Chronic administration into the PVN of the hypothalamus increased both carbohydrate and fat intake which can result in an increase in body weight gain and fat (Wilding et al., 1991).

The present study was undertaken to test the hypothesis that NPY immunoreactivity will respond to a high-fat diet.

In this experiment, the NPY immunoreactive varicosities did not have many changes except in the PeF of the mice fed with the high-fat diet for 1 week. It has been reported that the PeF is related to modulate sympathetic activity and is the primary hypothalamic site, which contains feeding-sensitive receptors mediated by NPY (Huang et al., 1998). The neurons of the PeF project to the medial preoptic area, plays an important role in the integration of cardiovascular and fluid regulation (Huang et al., 1998). It is likely that an increase in NPY immunoreactivity in mice fed high fat diet (Figure 3.3) may be due to low sympathetic activity reported previously (Matsuo et al, 1995).

In contrast to the above, after the mice fed a high-fat diet for 8 weeks, NPY immunoreactivity was significantly increased in the VMH, LH, and slightly increased in the DMH, and PVN.

It is known that NPY is one most potent stimulator of food intake. The present studies have also showed that there is a significant higher energy intake in the mice fed a high fat diet compared with the mice fed a low fat diet (Fig. 3.3). Therefore, it is possible increased NPY immunoreactivity found in this study may likely contribute to increased

food consumption in these mice. It is known that the LH and VMH are functionally opposite to each other, and that LH is a hunger centre, and that VMH is a satiety centre. Both increased NPY immunoreactivity in LH and VMH appears to conflict to each other. Indeed, the determinant of the functional significance of NPY is also on the receptor of NPY and the downstreams of that receptor. However, at the present stage, this study will not be able to answer these questions. These types of questions can be answered by using double labelling of the NPY receptors and possible down-stream neuromediators. The candidate neuromediators in LH and VMH regions are orexin and melanocortin concentrating hormone.

Furthermore, the analysis from my study shows that the percentage of increase in NPY-like immunoreactivity in the high fat diet was different in different hypothalamic areas (Table 4.2). Much greater increases were found in the VMH and LH, and in less degree in DMH and PVN. As it is known that VMH and LH largely affect appetite while DMH and PVN largely influence energy expenditure (Xin et al., 2000), it is possible that altered NPY immunoreactivity in the mice which were fed a high fat diet mainly influences energy intake and in a less degree in energy expenditure.

Hypothalamus	LF=(LF8-LF1)/LF1 (%)	HF=(HF8-HF1)/HF1 (%)	HF/LF
VMH	20	170	8.5
LH	50	130	2.6
DMH	40	70	1.8
PVN	5	20	4

Table 4.2: This table represents the NPY immunoreactive varicosities of mice which were fed with a low or high fat diet for 8 weeks. Abbreviations: LF1: the number of NPY immunoreactive varicosities found in the mice fed the high-carbohydrate diet for 1 week; LF8: the number of NPY immunoreactive varicosities found in the mice fed a low-fat diet for 8 weeks; HF1: the number of NPY immunoreactive varicosities found in the mice fed high-fat diet for 1 week; HF8: the number of NPY immunoreactive varicosities of the mice fed a high-fat diet for 8 weeks.

In conclusion, my study supports that NPY is involved in the regulation of energy balance. NPY immunoreactivity does not respond to a high-fat diet in 1 week, but significantly increased after 8 weeks of a high-fat diet. This is in consistent with the data showed previously that chronic NPY administration can increase amount of a high-fat diet (Wilding et al., 1991).

Chapter 5: α-MSH immunoreactivity

5.1 Experiment Design

1.Assignation of treatment groups and aims:

Twelve, 3 weeks old, C57BL/6J mice were obtained from the Animal Resources Centre and fed a standard laboratory chow diet for 1 week to accommodate to the new environment. Afterwards, they were separated into four groups: the mice fed with a high-fat diet for 1 week and 8 weeks, the mice fed with a low-fat diet for 1 week and 8 weeks.

Aim: To compare the α-MSH immunoreactive varicosities in the hypothalamus of mice fed with a high-fat diet and low-fat diet after 1 week and 8 weeks.

2. Comparisons:

Group 1 vs group 2: the mice fed with a low-fat diet for 1 week versus the mice fed with a high-fat diet for 1 week.

Group 3 vs group 4: the mice fed with a low-fat diet for 8 weeks versus the mice fed with a high-fat diet for 8 weeks.

5.2 Materials

1. Histology

The mice were sacrificed by an overdose of abdominal injection of Nembutal (120mg/kg). The mice were perfused with 0.9% NaCl 100ml and followed by 4% Paraformaldehyde 200ml. Brains were dissected out and stored in 4% Paraformaldehyde

for 12 hours for post fixation. Brains tissues were then put into 30% sucrose for about 20 hours. They were then cut at a 25μm thickness by the cryostat machine in –17°C.

2. Antibody Dilution

In order to get the maximum contrast between positively stained boutons and background, the concentration of primary antibody for MSH was tested for the best concentration of 1:8,000.

5.3 Methods

1. Detection of α-MSH Immunoreactivity:

The immunohistochemistry technique was used in this study.

2. Negative control

Brain sections subject to immunostaining procedures in the absence of the primary antibody did not show any signals.

5.4 Results

Positive immunostaining for MSH was detected in all of the hypothalamic regions examined. Background staining remained low and MSH positive immunoreactivity cell bodies were easily detected in some areas such as LH under the microscope.

In the present study, the quantification of NPY like immunoreactivity was carried out only in the hypothalamus. Positively stained buttons and neurons were counted. The colours after stained for immunohistochemsitry against individual

antibodies appear to be light brown, deep brown and black. Only the black stained positively buttons were counted in this study.

Areas	LF fed mice		HF fed mice	
Hypothalamus	1 week (n=6)	8 weeks (n=6)	1 week (n=6)	8 weeks (n=6)
Arc#	259.7±11.1	217.2±28.0	329±22.7	364.3±9.0
VMH	327.5±31.3	422.5±66.2	345.8±33.2	333.8±60.1
LH*#	95.8±17.1	162.7±18.9	126.5±9.0	260.5±16.6
DMH*#	454±46.7	642±123.0	517±5.4	1026±101.0
PeF*	8.8±1.4	24.8±8.1	6.3±1.8	15.3±5.6
PVN*	162±12.6	243±6.6	184.8±10.8	222.8±14.9

Table 5.1. Distribution of MSH immunoreactive varicosities in the hypothalamus of mice fed low-fat diet were examined after 1 week and 8 weeks.
Means ± S.E.: *p=0.05 or less for age effect where values in a given area from the mice after 1 week of feeding were compared to those mice after 8 weeks of feeding by a two-way ANOVA; #p=0.05 or less for a diet effect where values in a given area from the LF fed mice were compared to those from the HF fed mice by a two-way ANOVA. Abbreviation: PVN: paraventricular hypothalamic nucleus; Arc: arcuate hypothalamic nucleus; VMH: ventromedial hypothalamic nucleus; LH: lateral hypothalamus; DMH: dorsomedial hypothalamic nucleus; PeF: perifornical nucleus

Group 1 vs group 2: The comparison was made in mice fed with a low-fat diet or a high-fat diet for 1 week.

P-values were presented in the following table for the number of MSH-immunoreactive varicosities counted in the hypothalamic regions of the hypothalamus.

Region	Arc	VMH	LH	DMH	PeF	PVN
P-value	0.0205	0.6962	0.1437	0.2110	0.2744	0.2015

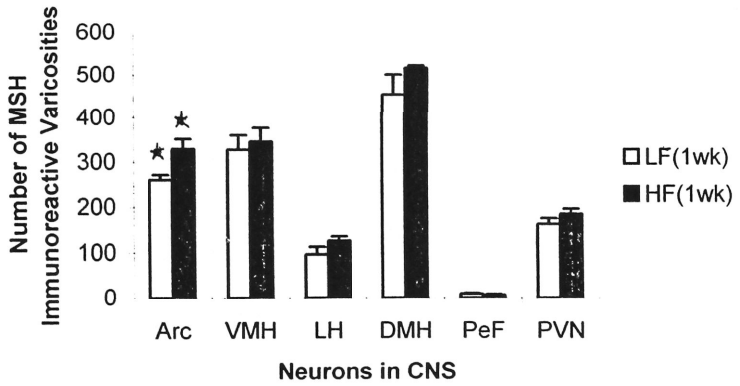

Figure 5.1: Comparison of a number of MSH-immunoreactive varicosities in the hypothalamic regions in the mice fed with a low-fat or a high-fat diet for 1 week. (Mean±SE, * denotes significant differences ($p<0.05$))

In Arc, the number of the MSH positive staining of the mice fed with a high-fat diet for 1 week was significantly (21%) greater than that of the mice fed with a low-fat diet for 1 week ($p<0.05$).

Group 3 vs group 4: the comparison was made between mice fed a low-fat or high-fat diet for 8 weeks.

P-values were presented in the following table for the number of MSH-immunoreactive varicosities counted in the hypothalamic regions of the mice hypothalamus.

Region	Arc	VMH	LH	DMH	PeF	PVN
P-value	0.0005	0.3448	0.0027	0.0366	0.3569	0.2361

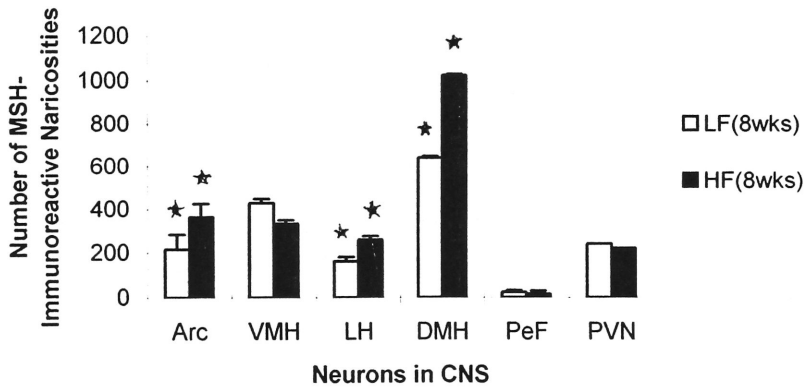

Figure 5.2: Comparison of a number of MSH positive boutons in the hypothalamic regions of the mice fed with a low-fat diet for 8 weeks vs the mice fed with a high-fat diet for 8 weeks. (Mean±SE, * denotes significant differences ($p<0.05$))

In Arc, the number of the MSH-immunoreactive varicosities of the mice fed with a high-fat diet for 8 weeks was significantly (40%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In DMH, the number of the MSH-immunoreactive varicosities of the mice fed with a high-fat diet for 8 weeks was significantly (37%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

In LH, the number of the MSH-immunoreactive varicosities of the mice fed with a high-fat diet for 8 weeks was significantly (38%) greater than that of the mice fed with a low-fat diet for 8 weeks ($p<0.05$).

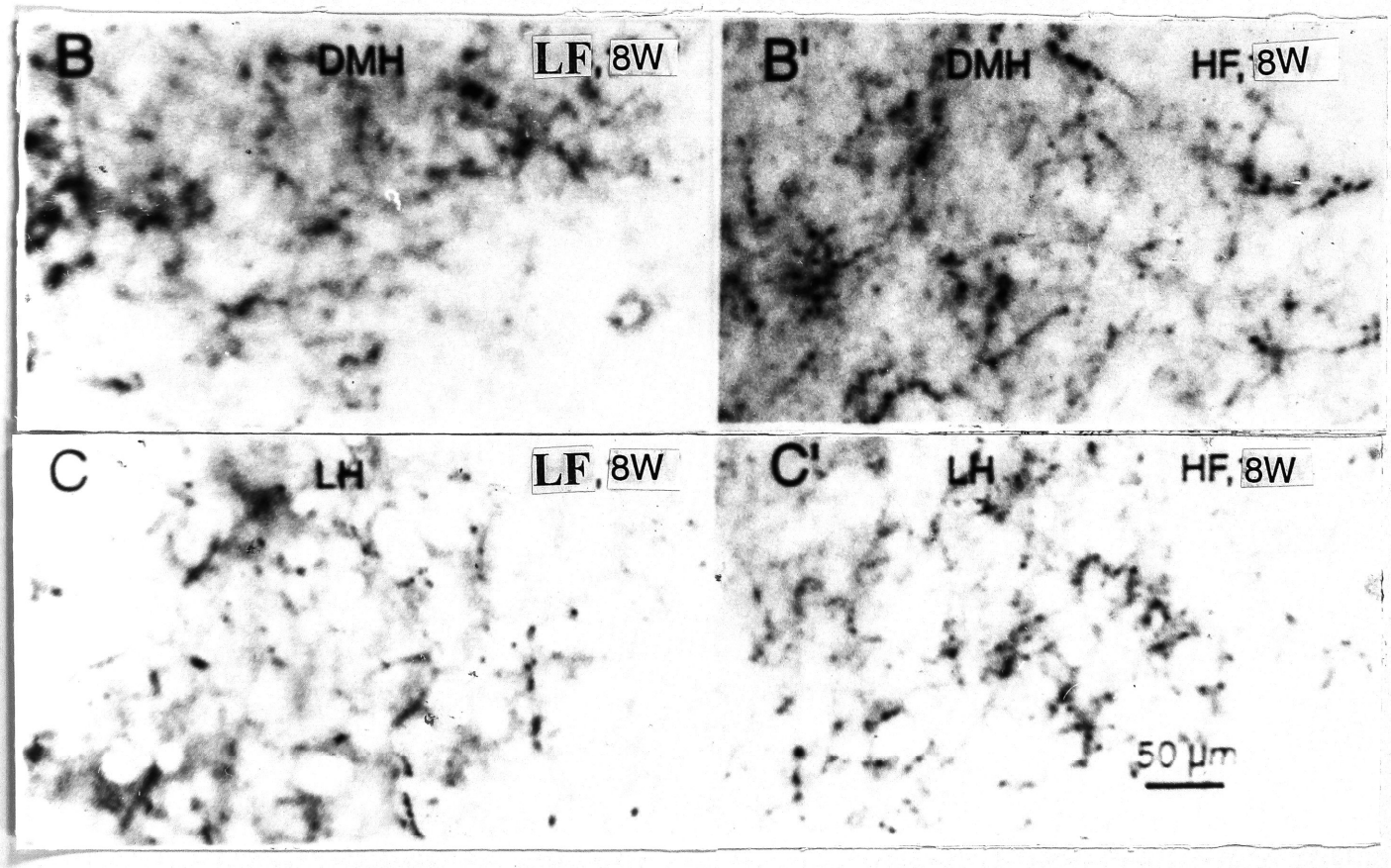
B
DMH
LF, 8W
B'
DMH
HF, 8W
C
LH
LF, 8W
C'
LH
HF, 8W
50 µm

Figure 5.3: Photomicrographs (B, B', C, C') of 25μm coronal sections of C57Bl/6J mouse brain immunohistochemical staining for the presence of α-MSH. B, C are from the mice fed with low-fat for 8 weeks. B', C' are from the mice fed with high-fat for 8 weeks. All sections are approximately at the level of bregma (-1.58) and photographs taken at 40X magnification. Pairs of sections (B, B', C, C') show increased numbers of positively stained varicosities in the mice fed with a high-fat diet for 8 weeks to the mice fed with a low-fat diet for 8 weeks. (B, B') dorsomedial hypothalamic area (DMH), (C, C') lateral hypothalamic area (LH).

5.5 Discussion

The α-MSH immunoreactive neurons are in the hypothalamus and brainstem. There is a large amount of α-MSH-like immunoreactivities found in the hypothalamus. These areas of the hypothalamus containing alpha-MSH immunoreactivity are largely involved in body weight control. These areas are the Arc, PeF and DMH. The Arc, where leptin receptor mRNA was found (Huang et al, 1996), is especially involved in the body weight control. In the following paragraph, I will firstly describe the α-MSH immunoreactivity in the hypothalamic nuclei of the mice fed with a high-fat diet for 1 week. Secondly, I will discuss the α-MSH immunoreactivity in these hypothalamic nuclei after the mice were fed with a high-fat diet for 8 weeks. Both will then be compared with the low-fat diet. Thirdly, I will discuss the duration of the high fat feeding, and how it influenced the alpha-MSH immunoreactivity.

After the mice were fed with a HF diet for 1 week, the only hypothalamic area that showed a significant increase in α-MSH immunoreactivity was the Arc. It is known that the majority of NPY neurons are located in Arc. It was described previously that NPY is a stronger stimulus which induces food intake, while α-MSH has the opposite effect when compared to NPY. α-MSH can reduce food intake, and the increased α-MSH contents may play a inhibitory role to the neurons in the Arc (Figure 5.1).

It is also known that the LH is a hunger centre which can stimulate food intake. The α-MSH can inhibit food intake and increase energy expenditure(Lu D., 1994). It is also involved in increasing sympathetic activity. My study showed that

the mice fed a high-fat diet for 8 weeks had low energy intake compared to the mice fed a low-fat diet. In this part of study, it was found that α-MSH was increased by 38% in the LH of the mice fed with a HF diet for 8 weeks. Therefore, it is possible that increased alpha-MSH-like immunoreactivity may contribute to low energy intake in mice fed a high fat diet (Figure 3.3).

In the present study, it was found that the DMH showed a large increase in α-MSH immunoreactive varicosities in this area. There was an increase in terminal buttons which were consistent with rich amounts of α-MSH. This indicates that there was innervation in this area when you are on a HF diet for 8 weeks. For the last three years it was apparent that the DMH plays an important role in body weight control and it seems that the DMH is a common centre between the Arc nucleus and the LH.

Furthermore, the DMH has large connections with the brain structure in the brainstem (Swanson L. W. et al., 1981). According to the results that recently came out from our laboratory, it was shown that in genetic obese mice, the DMH showed a large decrease in α-MSH immunoreactivity. It is known that these obese genetic mice had a much higher calorie intake compared with their counter parts. Therefore, all these suggest that a decrease in α-MSH contents in the DMH contributed to the increase in food intake. This is consistent with my studies. My results showed an increase in α-MSH immunoreactivity in the DMH, and this might contribute to the decreased food intake in the HF diet mice.

The results showed that mice, after being on a HF diet, featured more α-MSH immunoreactive cells in the hypothalamus nuclei. Those nuclei are the

Arc, LH and DMH. After plotting the differences on the Table 5.2, it can be seen that the DMH showed the most significant changes in the difference between 1 week and 8 weeks. The LH also showed these large and significant changes, but this is not the case for the VMH, Arc, PeF and PVN. This study showed that the DMH increased by 40% in α-MSH immunoreactivity (98% which is over twice as much as compared to the LF diet). This means there are diet effects involved. The proportion of increase in the HF diet group is much greater than that in the LF diet group (Table 5.2). Similar to the results of DMH, the LH also showed the similar changes, but to a lesser degree (1.6 times increased). However, the VMH showed an increase in α-MSH immunoreactivity after the mice were fed with a LF diet (30%), but in the mice fed with a HF diet, there was no increase in α-MSH immunoreactivity, indeed, there was a slight decrease. This indicates that the high-fat diet can decrease α-MSH immunoreactivity in the VMH.

Hypothalamus	LF=(LF8-LF1)/LF1 (%)	HF=(HF8-HF1)/HF1 (%)	HF/LF
Arc	20	10	0.5
LH	70	110	1.6
DMH	40	98	2.5
VMH	30	3	0.1

Table 5.2: The increased percentage of α-MSH immunoreactive varicosities of mice fed low-fat or high-fat for 8 weeks. *Abbreviations:* LF1: the number of MSH immunoreactive

varicosities of the mice fed low-fat diet for 1 week; LF8: the number of MSH immunoreactive varicosities of the mice fed low-fat diet for 8 weeks; HF1: the number of MSH immunoreactive varicosities of the mice fed high-fat diet for 1 week; HF8: the number of the MSH immunoreactive varicosities of the mice fed high-fat diet for 8 weeks.

Chapter 6 : Leptin receptor mRNA expression

6.1 Experiment Design

1.Assignation of treatment groups

Twelve, 3 weeks old, C57BL/6J mice were obtained from the Animal Resources Center and fed a standard laboratory chow diet for 1 week to accommodate to the new environment. Afterwards, they were separated into four groups: the mice fed with a high-fat diet for 1 week and 8 weeks, the mice fed with a low-fat diet for 1 week and 8 weeks.

Aim: To examine the levels of leptin receptor mRNA statement in the hypothalamus of mice fed a high-fat diet and low-fat diet for 1 and 8 weeks.

2. Comparisons:

Group 1 vs group 2: the mice fed with a low-fat diet for 1 week versus the mice fed with a high-fat diet for 1 week.

Group 3 vs group 4: the mice fed with a low-fat diet for 8 weeks versus the mice fed with a high-fat diet for 8 weeks.

6.2 Materials

1.Histology

The mice were sacrificed by an overdose of abdominal injection of Nembutal (120mg/kg). The mice were perfused with 0.9% NaCl 100ml and followed by 4% Paraformaldehyde 200ml. Brains were dissected out and stored in 4%

Paraformaldehyde for 12 hours for post fixation. Brains tissues were then put into 30% sucrose for about 20 hours. The brains were frozen in -80°C freezer. Then they were sectioned at 25μm thickness by freeze microtome in -17°C.

2. Leptin receptor probe and control probe:

Oligonucleotide probes were used here to detect the levels of leptin receptor mRNA statement. Control probes were also used as comparison for the examination. Sequences of each oligo probes were previously presented in the chapter 2 of this thesis.

6.3 Methods

1. Detection of Leptin Receptor

In situ hybridisation method was used to examine the statement of Leptin receptor mRNA.

2. Control

As a comparative control the Tubby probe was used because this probe has been demonstrated in our laboratory to consistently give strong localised signals. Further, additional brain sections were processed in the absence of leptin receptor probes which did not show any signal.

The distribution of signals was analysed using light microscope and the atlas of the mouse brain. Representative photographs of coronal sections were taken with Leica microphotographic apparatus and printed on AGFA Multigrade paper.

6.4 Results

Positive signals of leptin receptor mRNA statement were found in the piriform cortex (Pir), hippocampus, hypothalamic arcuate nucleus, and ventromedial hypothalamic nucleus.

Extensive signals of Tubby mRNA were found throughout virtually all areas of the mouse brain. The present study showed that there were no differences in the level of leptin receptor mRNA statement between the mice fed a high and low fat diet for 1 week and 8 weeks.

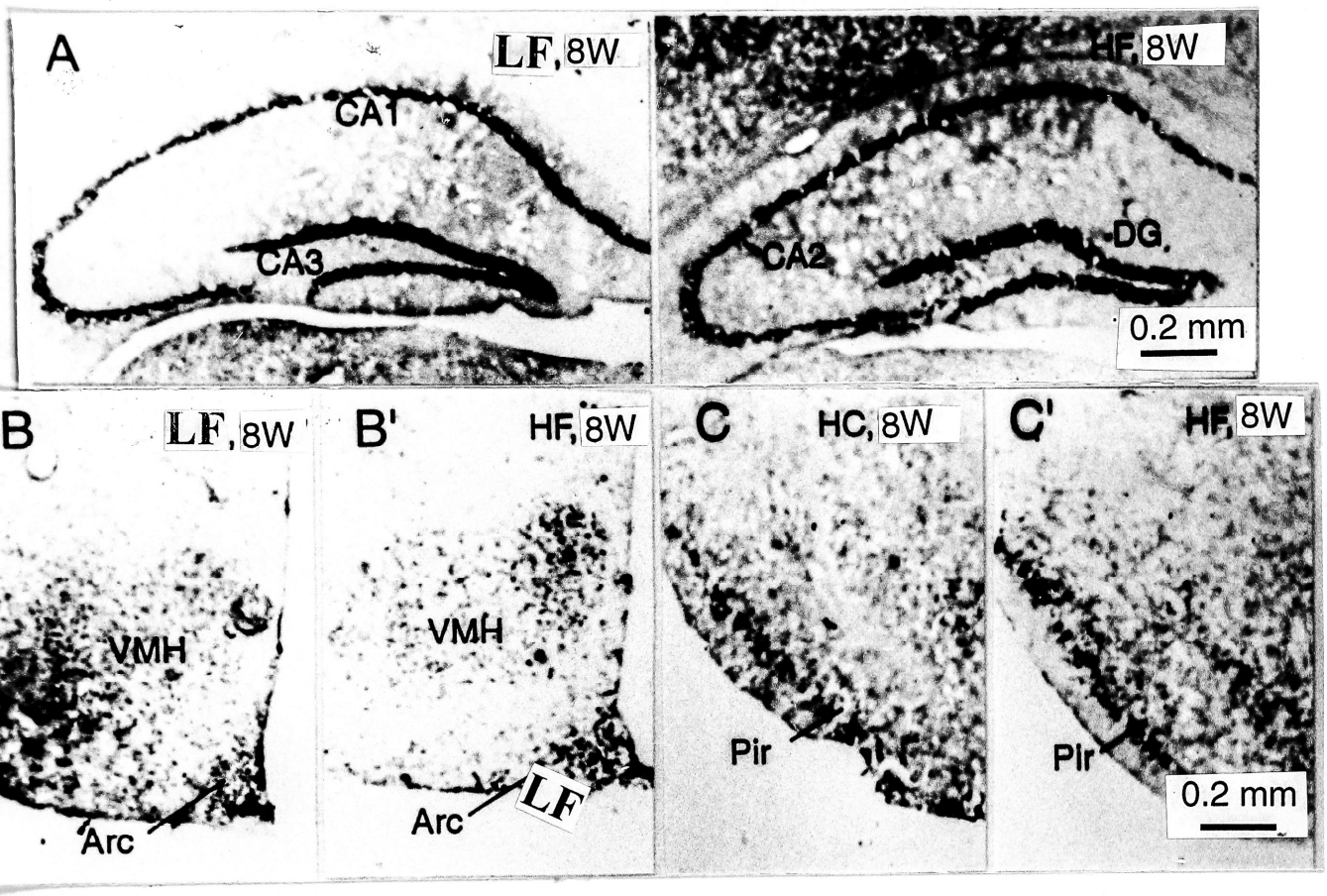
A
LF, 8W
CA1
CA3
A'
HF, 8W
CA2
DG
0.2 mm
B
LF, 8W
VMH
Arc
B'
HF, 8W
VMH
Arc
LF
C
HC, 8W
Pir
C'
HF, 8W
Pir
0.2 mm

Figure 6.1: Photomicrographs (A, A', B, B', C, C') of 25 μm coronal sections from C57Bl/6J mouse brain. Brain sections were processed for the detection of leptin receptor mRNA statement. A, B, C are from the mice fed with low-fat diet for 8 weeks and A', B', C' are from the mice fed high-fat for 8 weeks. All sections are approximately at the level of bregma (-1.58) and the photographs were taken at 10X magnification. (A, A') fields Cal1-3 of ammons horn (CA1-3), (B, B') arcuate nucleu (Arc), (C, C') pirifornical cortex (Pir).

6.5 Discussion

It has been known that leptin and the leptin receptor system play a very important role in the regulation of body weight control. Leptin is produced in the adipocyte, secreted in the blood stream, and then transported to the hypothalamus which signals the brain network, especially NPY and alpha-MSH system, to regulate body weight. This regulation has been highly suggested also to regulate the sympathetic, lipogenic and lipolytic function (Baskin D.G. et al, 1999; Thornton J.E. et al, 1997).

In this part of my study, leptin mRNA expression in the hypothalamus of mice fed a high and low fat diet, was demonstrated. The results showed that the level of expression has no detectable difference in the hypothalamus across all four groups. These four groups are mice which were fed a high or low fat diet for one week and eight weeks. In agreement with my college, Dr. Lin and I have found that leptin resistance occurs only after a long-term HF diet, but not before 18 weeks of a HF diet. Therefore, this support the concept that at early stages there is no central leptin resistance (Lin S. et al, 2000; Pickavance L.C. and Widdowson et al, 1999). The concept was first reported by Van Heek, M (Margaret, 1997). In their studies, Van Heek found at early stages of a high-fat diet the central nervous system was very sensitive to the intracerebroventricular administration of leptin. Similar results were given by Widdowson (Widdowson P.S. et al., 1998). These experiments showed that food intake was reduced and that body weight was reduced as well as body fat stores were reduced after the central administration of leptin. These experiments suggested at the early stages, that the brain was sensitive to the leptin.

However, after eating a high fat diet for very long period of time (eg: 18 weeks), central leptin resistance can occur.

From technical point of view, the non-radioactive in situ hybridisation method is less sensitive in terms of how it quantifies the amount of mRNA statement compared to the radioactive in situ hybridisation. Therefore, it is likely that there are the differences between the mice fed high and low fat diets. However, non-radioactive in situ hybridisation is unable to demonstrate the subtle changes. On the other hand, non-radioactive method provides a safe environment without isotope contamination and is easy to handle. Nevertheless, I would suggest after my study that the radioactive in situ hybridisation method should be used in the study of leptin receptor mRNA statement in diet-induced obese mouse model.

Further, as I have described in the literature review, there are different leptin receptor isoforms, all of which possess different functions. Furthermore, in my study, the probes that I have used for the detection of leptin receptor mRNA statement are derived from the conservative regions of leptin receptor mRNA. This means that the leptin receptor mRNA statement demonstrated in my study represents all isoforms without the specificity in identifying subtypes of leptin receptor mRNA. Therefore, it is possible that changes may occur at a sub-type level rather than at the level of all isoforms. Therefore, I would like to suggest to that it is necessary to examine individual isoforms of the leptin receptor in future study.

Chapter 7: Overall Discussion and References

7.1 Overall discussion

The present study has examined the levels of hypothalamic c-fos, NPY, alpha-MSH and leptin receptor in mice fed high and low fat diets for 1 and 8 weeks. It is demonstrated in this study that hypothalamus plays a very important role in the regulation of body energy balance. Overall, my study has demonstrated that not all regions of the hypothalamus show changes following a high fat diet. The areas showing the most changes in c-fos activity are lateral hypothalamus, ventromedial hypothalamic nucleus, dorsomedial hypothalamic nucleus, and paraventricular hypothalamic nucleus. The areas showing the most changes in NPY-like immunoreactivity are VMH. The areas showing the most changes in alpha-MSH are Arc. No changes were found in leptin receptor mRNA statement in the hypothalamus of mice fed a high and low fat diet for 1 and 8 weeks.

General speaking, among all markers examined in this study including c-fos, NPY, alpha-MSH and leptin receptor, hypothalamic c-Fos-like immunoreactivity shows significant changes after mice were fed a high-fat diet for 1 week. However, only very small changes were found in NPY-like immunoreactivity and virtually no changes in alpha-MSH and leptin receptor after mice were fed a high fat diet for 1 week. These results indicate that other neuromodulators were involved in the regulation of energy balance besides the NPY and alpha-MSH. The c-fos results suggest those neuromodulators are primarily located in the lateral hypothalamus and arcuate hypothalamic neucleus, which need further investigation.

In the C-fos experiment, the C-fos immunoreactivity had increased most significantly in LH of the mice which were fed a HF diet for one week, and the most striking changes in C-fos immunoreactivity were found in the VMH of the mice which were fed a HF diet for eight weeks. Therefore, the proportion of increased C-fos immunoreactivity in a HF diet group is much greater than that of a LF diet group (15 times). It was seen that the overall calorie intake was decreased, and this supports the notion that VMH is a satiety centre.

In the NPY experiment, it was found the increased NPY immunoreacitivty in VMH, LH of the mice fed with high-fat diet after 8weeks. It is known that NPY is one of the most potent stimulators of food intake. The greater increase was found in the VMH and LH which effect appetite and to a less degree in DMH and PVN which influences energy expenditure. This indicated that a high-fat diet mainly influences energy intake and in less degree in energy expenditure in this study.

In the α-MSH experiment, it was shown that DMH, and LH have the most significant increases in the mice after they were fed a HF diet after 8 weeks. This means there are diet effects involved. However, the VMH showed an increase in α-MSH immunoreactivity after the mice were fed with a LF diet and there was a decrease in α-MSH immunoreactivity in the HF dietary mice. This indicates the high-fat can decrease α-MSH imunoreactivity in VMH.

	C-Fos		NPY		α-MSH	
	D	T	D	T	D	T
VMH	15	15.8	8.5	32.5	0.1	4
LH	0.2	0.2	2.6	10	1.6	2
DMH	0.15	0.3	1.8	4	2.5	6
Arc	0.6	2.1	1.9	3.5	0.5	2.3

Table 7.1: The interaction of time and diet which effect the FOS, NPY, MSH immunoreactivity. The letter D (D = ((HF8-HF1)/HF1)/((LF8-LF1)/LF1) indicates the diet effects for mice after 8 weeks of feeding. The letter T ((HF8-LF8)/LF8)/((HF1-LF1)/LF1) indicates the time effects between mice fed for either 1 week or 8 weeks on a HF diet. Abbreviations: LF1: the number of immunoreactivity of the mice fed low-fat for 1 week; LF8: the number of immunoreactivity of the mice fed low-fat for 8 weeks; HF1: the number of immunoreactivity of the mice fed high-fat for 1 week; HF8: the number of immunoreactivity of the mice fed high-fat for 8 weeks.

VMH is a satiety centre. Duration of high fat feeding can have greater influences in the satiety center (VMH). The present study showed the longer period of high fat feeding the greater increases in c-Fos and NPY-like immunoreactivities, and decreases in alpha-MSH-like immunoreactivity in VMH (Fig. 7.1). Opposing effects between NPY and alpha-MSH found in this study support the concept that NPY and alpha-MSH are an important pair of neurotransmitters in the hypothalamus for regulating body weight.

Although this phenomenon can also be seen in other areas such as in lateral hypothalamus and arcuate hypothalamic nucleus, but it is in much less of a magnitude

7.2 Implications for the future study

It is clear that more studies are needed to be carried out to understand how the hypothalamus responds to a high fat diet in regulation of energy balance. Especially the long term feeding of high fat diet should be investigated, so as to examine the possible development of central leptin resistance in diet induced obesity. More importantly, further study should examine specific receptors of NPY and alpha-MSH.

For example, NPY-Y5 receptor has recently been suggested to be a feeding receptor and melanincortine concentrating hormone receptor subunit 4 (MC4), these play a key role in transmitting Arc-alpha-MSH projection into PVN. Finally, the number of mice used in this study should be increased to 6 per group for processing histology, which is beyond the time limitation of my one year project.

7.3 General conclusions

In summary, this study has provided preliminary data which showed the changes of hypothalamic c-fos, NPY, alpha-MSH, and leptin receptor levels in mice fed high fat diet for both 1 and 8 weeks. The hypothalamus plays an important role in the regulation of energy balance in relation to high energy intake as demonstrated in a high-fat diet in this study. VMH, LH, DMH and Arc are the key hypothalamic areas involved in the energy balance regulation.

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